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(54) **Title:** NUCLEIC ACID SENSOR MOLECULES AND METHODS OF USING SAME

(57) **Abstract:** Methods for engineering a nucleic acid sensor molecule are provided. Biosensors comprise a plurality of nucleic acid sensor molecules labeled with a first signaling moiety and a second signaling moiety. The nucleic acid sensor molecules recognizes target molecules which do not naturally bind to DNA. Binding of a target molecule to the sensor molecules triggers a change in the proximity of the signaling moieties which leads to a change in the optical properties of the nucleic acid sensor molecules on the biosensor. Reagents and systems for performing the method are also provided. The method is useful in diagnostic applications and drug optimization.

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NUCLEIC ACID SENSOR MOLECULES
AND METHODS OF USING SAME

5

Field of the Invention

The invention relates generally to nucleic acids and more particularly to nucleic acid sensor molecules containing a catalytically active domain that is modulated upon recognition of a target by a target modulation domain of the nucleic acid sensor molecule.

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Background of the Invention

In addition to carrying genetic information, nucleic acids can adopt complex three-dimensional structures. These three-dimensional structures are capable of specific
15 recognition of target molecules and, furthermore, of catalyzing chemical reactions. Nucleic acids will thus provide candidate detection molecules for diverse target molecules, including those which that do not naturally recognize or bind to DNA or RNA.

A nucleic acid which binds to a non-nucleic acid target molecule through non-Watson-Crick base pairing is termed an aptamer. In aptamer selection, combinatorial
20 libraries of oligonucleotides are screened *in vitro* to identify oligonucleotides, or aptamers, which bind with high affinity to pre-selected targets. Both small biomolecules (*e.g.*, amino acids, nucleotides, NAD, S-adenosyl methionine, chloramphenicol), and large biomolecules (thrombin, Ku, DNA polymerases) are effective targets for aptamers. Aptamer biosensors have been used to detect specific analyte molecules. For example, fluorescently labeled
25 anti-thrombin aptamers attached to a glass surface have been used to directly detect the presence of thrombin proteins in a sample by detecting changes in the optical properties of the aptamers (Potyrailo, et al., 1998). In this method continuous binding of thrombin to the labeled aptamer is requisite for detection to occur, since the concentration of thrombin in a test sample is monitored by directly detecting fluorescent emission of the aptamer-ligand
30 complex. Another method of detecting binding of a ligand to an aptamer has also been described which relies on the use of fluorescence-quenching pairs whose fluorescence is sensitive to changes in secondary structure of the aptamer upon ligand binding (Stanton, et al. 2000) to form a fluorescent aptamer-ligand complex, and again, continuous binding of the ligand to the aptamer is required for signal generation and, hence, for detection to occur.
35 A limitation with this type of aptamer-derived biosensor is that ligand-mediated changes in

secondary structure were engineered into the aptamer molecule via a laborious engineering process in which four to six nucleotides were added to the 5' end of the aptamer that was complementary to the bases at the 3' end of the thrombin binding region. In the absence of thrombin, this structure forms a stem loop structure, while it forms a G-quartet structure in

5   the presence of thrombin. Fluorescent and quenching groups attached to the 5' and 3' end signal this change. In aptamer-based detection without the use of amplification steps, assay sensitivity and hence the limit of detection is set empirically by the affinity of the aptamer-ligand complex, the K_D value. Using aptamer-based sensor molecules one can detect analyte binding in both solution (homogeneous) and on solid supports (heterogeneous).

10   Other ligand detection methods known in the art are based upon antibody binding. Similar to the aptamer-based methods, antibody-based detection requires continuous ligand binding and ligand-antibody complex formation for the generation of a detectable signal. In addition, antibody methods such as ELISA or competitive RIA, while robust, are restricted in utility because these methods require that heterogeneous assay conditions be employed:

15   1] detection is done on a solid surface; 2] in most applications both a capture antibody and detection antibody are required; 3] for ELISA-based protein detection methods, the antibodies must recognize the folded, native structure of the protein that is present in cell or tissue isolates and; 4] antibody and protein based detection methods have not been described for intracellular or *in vivo* based analyte detection. That antibodies have not been

20   employed for intracellular and *in vivo* based detection of proteins, drugs or metabolites is due to several technological factors. First monoclonal antibody fragments are unstable and do not fold properly when expressed as intracellular protein molecules. Second, intracellular detection requires homogeneous assay formats and these solution-based detection methods require the sensor to have a ligand sensing or modulation domain

25   coupled directly to a catalytic or signal generating region of an enzyme or catalytic biomolecule. A fundamental and important consequence of the limitations of antibody-based detection methods is that they can not function as a universal reagent for all assays and tests that can be employed in drug discovery and development. These assays include 1] the initial discovery of a drug target through protein or metabolite profiling, 2] the

30   subsequent use of that same drug target in the discovery of drug leads through high throughput screening and, 3] the optimization of drug leads against that same drug target through an evaluation of lead efficacy in mechanistic cellular and *in vivo* animal assays.

To streamline the drug discovery and development process and improve the efficiency of evaluating drug targets and drug leads, detection reagents are needed that can

function in a context-independent manner. Needed then is a molecular sensor that can function in multiple assay environments and in multiple assay formats. The present invention is generally drawn to nucleic acid molecular sensors that can function in environments and formats that includes but are not limited to solution-based detection

5   (homogeneous *in vitro* biochemical assays or *in vivo* cellular and animal assays), chip-based (heterogeneous *in vitro* assays on solid surfaces), and assays in complex biological isolates from blood plasma, cell lysates or tissue extracts.

Nucleic acid-based detection schemes have exploited the ligand-sensitive catalytic properties of some nucleic acids, *e.g.*, such as ribozymes. Ribozyme-based prototype

10   nucleic acid sensor molecules have been designed both by engineering and by *in vitro* selection methods. Engineering methods exploit the apparently modular nature of RNA structures; these sensors couple molecular recognition to signaling by simply joining individual target-modulation and catalytic RNA domains through a double-stranded or partially double-stranded RNA linker. ATP sensors, for example, were created by

15   appending the previously-selected, ATP-binding aptamer-derived sequences (Sassanfar and Szostak, 1993) to either the self-cleaving hammerhead ribozyme (Tang and Breaker, 1997) or the L1 self-ligating ribozyme (Robertson and Ellington 2000). Robertson and Ellington (2000) have demonstrated that the enzymatic activity of a ligase ribozyme (derived from the L1 ligase described in Robertson and Ellington (1999)) can be modulated by a small

20   molecule ligand, or small molecule target recognition. In this case, the ligase ribozyme can be employed as a nucleic acid sensor molecule and used to detect the presence and level of its cognate ligand by monitoring the ligation of a small, labeled second oligonucleotide substrate on to the ribozyme. A distinct feature of this detection method is that the actual detection event, *e.g.*, monitoring oligonucleotide substrate ligation to the ribozyme, occurs

25   after the ligand interacts with the nucleic sensor molecule. Hence, unlike antibody, or aptamer based detection methods, the ribozyme-based ligand detection method of Robertson and Ellington does not require continuous binding of the ligand to the sensor molecule in order to generate a detectable signal. In a complementary approach, radiolabeled hammerhead ribozymes which undergo cleavage upon binding to a ligand, have been used

30   to detect ligand by monitoring the release of the label from the ribozyme (Soukup, et al., 2000, and Breaker, 1998). Limitations of the use of ligand modulated hammerhead ribozymes described by Soukup, et al., 2000, and Breaker, 1998 include: 1] the need for a two-step detection method for determining the enzymatic activity of the surface-immobilized hammerhead-derived sensors; 2] the need for radiometric determination of

hammerhead activity in both solution and solid-surface based assay formats; 3] the need for significant chemical and structural modification of the hammerhead-based biosensor to render them suitable for optical based detection methods.

Another limitation of the engineering method to sensor generation is that it has been generally thought not to be applicable to the development of protein-dependent ribozymes. Robertson and Ellington (2001) describe their own efforts to extend this methodology to the identification of protein and peptide-dependent ribozymes, but state that simply appending aptamer-derived sequences to the catalytic domain of the L1 ligase at stem C, yields little or no target dependent modulation. Furthermore, the authors state that the "principles required for engineering protein-dependent ribozymes must be fundamentally different from those for identifying ribozymes dependent on small-molecules." Hence, in order to identify protein and peptide dependent ribozymes, Ellington and Robertson undertook a laborious *in vitro* selection process which involved randomization of the catalytic core of the L1 ligase coupled with multiple rounds of positive and negative selection. Ellington and Roberts (2000) describe several limitations of the ligase-derived sensors that they developed. First, the nucleoprotein enzymes developed by Ellington and Robertson (2000) required a laborious *in vitro* selection process to identify peptide and protein dependent ligases. Secondly, Ellington and Roberts (1999) describe a region of the L1 ligase that is required for allosteric ribozyme function, termed the effector oligonucleotide binding domain. It was postulated that the effector oligonucleotide binding domain of the ligase formed complementary base pairing interactions with the oligonucleotide substrate binding site, driving the ribozyme into an inactive conformation. The effector oligonucleotide, when added to the L1 ligase activates (k_{act}) the enzyme by over 10,000 fold over the L1 ligase reaction measure in the absence of effector (k_{unact}). Hence, the native L1-ligase has a switch factor (k_{act}/k_{unact}) greater than 10,000, which determines the sensitivity of a ribozyme-based detection method. When the effector oligonucleotide binding domain of the L1 ligase is deleted, the ligase activity of the deletion mutant is only 3-5 fold lower than the ligase activity of L1 ligase with the effector oligonucleotide bound to the effector oligonucleotide binding domain (Ellington and Robertson (1999). This indicates that L1 ligases deleted of the effector oligonucleotide binding domain may not be not subject to further allosteric regulation. Hence, a hindrance to the development of L1 ligase-based biosensor technology is the lack of a general method for the generation of biosensors that can work in multiple assay and detection formats required of solution-based and chip-based

biosensors and, those that can work in multiplexed formats and in complex biological extracts.

Summary of the Invention

The nucleic acid sensor molecules of the present invention are used to monitor the

5   presence or concentration of various target molecules (proteins, post-translationally modified forms of proteins, peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, and ions) and function in solution-based homogeneous assays using optical or other detection methods; in solution-based homogeneous assays using PCR-based or other nucleotide amplification-based detection

10   methods; in homogeneous intracellular assays using PCR-based detection or other nucleotide amplification-based detection methods; in heterogeneous assays (surface-immobilized nucleic acid sensor molecules and surface-capture nucleic acid sensor molecules) using optical detection methods; and in heterogeneous assays (surface-immobilized nucleic acid sensor molecules and surface-capture nucleic acid sensor

15   molecules) using PCR-based detection or other nucleotide amplification-based detection methods. And finally the nucleic acid sensor molecules of the present invention function in formats where the target analytes are present in complex biological mixtures, or the assays are themselves performed in multiplexed formats.

The nucleic acid sensor molecules of the present invention were developed through

20   a combination of engineering and selection methods that are now shown to be useful for identifying nucleic acid sensor molecules against a wide variety of target molecules including proteins (including specific post-translationally modified forms of proteins) peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, ions, carbohydrates, glycoproteins, hormones, receptors, antibodies, viruses,

25   transition state analogs, cofactors, dyes growth factory nutrients, etc.

In one embodiment of the invention the nucleic acid sensor molecules are based on cis-cleaving hammerhead ribozymes that have been designed to work as optical signaling molecules in a homogeneous assay format, and utilize fluorescence and FRET based methods of signal generation and detection.

30   In one embodiment of the invention nucleic acid sensor molecules are based on cis-cleaving hammerhead ribozymes that have been designed to work as optical signaling molecules affixed to a solid-support, and utilize fluorescence and FRET based methods of signal generation and detection.

In one embodiment of the invention the nucleic acid sensor molecules are based on cis-cleaving hammerhead ribozymes that have been designed to work as optical signaling molecules affixed to a solid-support and utilize surface plasmon resonance methods of signal generation and detection.

5   In one embodiment of the invention the nucleic acid sensor molecules are based on a 3-piece L1 ligase ribozyme that retains the effector oligonucleotide binding domain, and has been designed to detect target molecules, as a function of oligonucleotide substrate ligation to the nucleic acid sensor molecule, in solution using quantitative PCR-based methods.

In one embodiment of the invention the nucleic acid sensor molecules are based on a 10   2-piece L1 ligase ribozyme that couples the effector oligonucleotide substrate to the oligonucleotide substrate forming an oligonucleotide supersubstrate and, has been designed to detect target molecules, as a function of oligonucleotide supersubstrate ligation to the nucleic acid sensor molecule, in solution using quantitative PCR-based methods.

In one embodiment of the invention the nucleic acid sensor molecules are based on a 15   1-piece L1 ligase ribozyme that deletes the effector oligonucleotide binding domain. The 1-piece ligase is designed to self-ligate or circularize by joining the 3'-OH end and the PPP-5'-end of the ligase and detects target molecules, as a function of circularization of the nucleic acid sensor molecule, in solution using PCR-based methods.

In one embodiment of the invention the nucleic acid sensor molecules are based on a 20   3-piece L1 ligase ribozyme that retains the effector oligonucleotide binding domain, and has been designed to detect target molecules, as a function of oligonucleotide substrate ligation to the nucleic acid sensor molecule immobilized on a solid support and where the detection uses fluorescence-based methods.

In one embodiment of the invention the 3-piece L1 ligase nucleic acid sensor 25   molecules detect target molecules, as a function of oligonucleotide substrate ligation to the nucleic acid sensor molecule in solution and then are subsequently captured on a solid support and detected using fluorescence-based methods.

In one embodiment of the invention the 3-piece L1 ligase nucleic acid sensor molecules detect target molecules, as a function of oligonucleotide substrate ligation to the 30   nucleic acid sensor molecule in solution and then are subsequently captured on a solid support and detected using radiometric-based methods.

In one embodiment of the invention the nucleic acid sensor molecules are based on a 2-piece L1 ligase ribozyme and has been designed to detect target molecules, as a function of oligonucleotide substrate ligation to the nucleic acid sensor molecule immobilized on a

solid support and where the detection uses optical methods such as fluorescence-based methods.

In one embodiment of the invention the 2-piece L1 ligase nucleic acid sensor molecules detect target molecules, as a function of oligonucleotide substrate ligation to the

5   nucleic acid sensor molecule in solution and then are subsequently captured on a solid support and detected directly using fluoresence-based methods.

In one embodiment of the invention the 2-piece L1 ligase nucleic acid sensor molecules detect target molecules, as a function of oligonucleotide substrate ligation to the nucleic acid sensor molecule in solution and then are subsequently captured on a solid

10   support and directly detected using radiometric-based methods.

In one embodiment of the invention the nucleic acid sensor molecules is based on a 1-piece L1 ligase ribozyme that is transfected into a mammalian cell. The 1-piece ligase is designed to self-ligate or circularize by joining the 3'-OH end and the PPP-5'-end of the ligase and detects intracellular target molecules, as a function of circularization of the

15   nucleic acid sensor molecule. The circularized ligase molecules are then re-isolated from the cellular lysate and the amount of target present in the cell is quantified using solution based nucleotide amplification methods.

In one aspect, the invention includes a nucleic acid sensor molecule which includes a target modulation domain which recognizes a target molecule, a linker domain, a catalytic

20   domain, and an optical signal generating unit.

In one embodiment, the nucleic acid sensor molecule of the invention has an optical signal generating unit that includes at least one signaling moiety. In another embodiment, the nucleic acid sensor molecule of the invention has an optical signal generating unit which includes at least a first signaling moiety and a second signaling moiety. In another

25   embodiment, the first and second signaling moieties change proximity to each other upon recognition of a target by the target modulation domain. In another embodiment, the first and second signaling moieties include a fluorescent donor and a fluorescent quencher, and recognition of a target by the target modulation domain results in an increase in detectable fluorescence of said fluorescent donor. In another embodiment, the first signaling moiety

30   and said second signaling moiety include fluorescent energy transfer (FRET) donor and acceptor groups, and recognition of a target by the target modulation domain results in a change in distance between said donor and acceptor groups, thereby changing optical properties of said molecule.

In another embodiment, the invention includes a nucleic acid sensor molecule where the optical signal generating unit consists essentially of a first signaling moiety which changes conformation upon recognition of a target by the target modulation domain, thereby resulting in a detectable optical signal.

5   In yet another embodiment, the nucleic acid sensor molecule includes at least one modified nucleic acid.

In still another embodiment, the catalytic domain of the nucleic acid sensor molecule includes an endonucleolytic ribozyme. The endonucleolytic ribozyme can be, for example, a *cis*-endonucleolytic ribozyme or a *trans*-endonucleolytic ribozyme. In one embodiment,

10   the endonucleolytic ribozyme is a hammerhead ribozyme.

In another embodiment, the catalytic domain of the nucleic acid sensor includes a self-ligating ribozyme. The elf-ligating ribozyme can be, for example, a *cis*-ligase ribozyme or a *trans*-ligase ribozyme. The self-ligating ribozyme can be, e.g., a 1-piece ligase, 2-piece ligase or 3-piece ligase.

15   In another embodiment, the target modulation domain recognizes a target that is selected from proteins, post-translationally modified forms of proteins, peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, ions, carbohydrates, polysaccharides, hormones, receptors, antigens, antibodies, viruses, metabolites, co-factors, drugs, dyes, nutrients, or growth factors.

20   In one embodiment, the target modulation domain recognizes a protein or a post-translationally modified protein.

In another embodiment, the target modulation domain recognizes a post-translationally modified protein, wherein the post-translational modification can be a phosphorylation, prenylation, glycosylation, methionine removal, N-acetylation, acylation,

25   acylation of cysteines, myristoylation, alkylation, ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycoslylation, deamination of glutamine and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation, or lipidation.

In some embodiments, the target is a protein kinase. In other embodiments, the

30   target is a phosphorylated protein kinase. The phosphorylated protein kinase can be a monophosphorylated protein kinase or a diphosphorylated protein kinase. In one embodiment, the target protein is ERK, such as ERK1 or ERK2. In other embodiments, the post-translationally modified protein is ppERK, such as such as ppERK1 or ppERK2..

In other embodiments, the nucleic acid sensor molecule includes a target modulation domain which recognizes a component of a MAP kinase pathway, a product of a MAP kinase pathway, a MAP kinase pathway associated protein, or an extracellular component of a MAP kinase pathway. In one embodiment, the target modulation domain recognizes a

5   component of the ERK1/2 MAP kinase pathway, the JNK MAP kinase pathway, or the P38 MAP kinase pathway.

In some embodiments, the target modulation domain recognizes an endogenous form of a MAP Kinase (MEK), an endogenous form of a MAP Kinase Kinase (MEKK), or an endogenous form of MAP Kinase Kinase Kinase, (MEKKK). In other embodiments, the

10   target modulation domain recognizes an endogenous form of RAF kinase. In still other embodiments, the target modulation domain recognizes a Ras protein, a phosphatase, s a GTP binding protein, a GPCR, a cytokine, a growth factor, a cellular metabolite, or a small molecule.

In some embodiments, the nucleic acid sensor molecule includes RNA, DNA, or

15   both RNA and DNA.

The invention also relates to compositions containing a nucleic acid sensor molecule of the invention and a buffer. In some embodiments, the invention includes a composition containing a nucleic acid sensor molecule and a tissue extract, a cell extract or an *in vitro* cell culture. In other embodiments, a composition of the invention also includes an RNase

20   inhibitor, such as, for example, Va-riboside, vanadyl, tRNA, polyU, RNaseIn or RNaseOut. In some embodiments, a composition of the invention is substantially RNase-free.

The invention also relates to a composition which includes at least one nucleic acid sensor molecule affixed to a substrate. In some embodiments, the substrate is glass, gold or other metal, silicon or other semiconductor material, nitrocellulose, nylon, or plastic. In

25   particular embodiments, the nucleic acid sensor molecule is covalently attached to the substrate. In other embodiments, the nucleic acid sensor molecule is non-covalently attached to the substrate. In some embodiments, the nucleic acid sensor molecule is immobilized to the substrate *via* hybridization of a terminal portion of the nucleic acid sensor molecule to an oligonucleotide that is bound to the surface of the substrate.

30   In some embodiments, a composition includes a plurality of nucleic acid sensor molecules immobilized to the substrate via hybridization of a terminal portion of the nucleic acid sensor molecule to an array of oligonucleotides bound to the substrate at spatially discrete regions. In some embodiments, at least two members of this plurality each recognize different target molecules. The substrate can include, for example, at least 50

nucleic acid sensor molecules. In other embodiments, the substrate includes at least 250 nucleic acid sensor molecules, at least 500 nucleic acid sensor molecules, or at least 5000 nucleic acid sensor molecules.

In some aspects, the invention includes a system for detecting a target molecule which includes a composition according to the invention and a detector in optical communication with the composition, where the detector detects changes in the optical properties of the composition. In some embodiments, the system further includes a light source in optical communication with the composition. In some embodiments, the system also includes a processor for processing optical signals detected by the detector.

In one embodiment, the system for detecting a target molecule includes a plurality of nucleic acid sensor molecules where at least two of the biosensor molecules each recognize different target molecules.

In another aspect, the invention includes a method of identifying or detecting a target molecule in a sample by contacting a sample suspected of containing a target molecule with a nucleic acid sensor molecule of the invention, wherein a change in the signal generated by the optical signal generating unit indicates the presence of target in the sample. In some embodiments, the method further includes quantifying the change signal generated by the optical signal generating unit to quantify the amount of target molecule in the sample. In some embodiments, the sample is an environmental sample, biohazard materials, organic samples, drugs and toxins, flavors and fragrances, and biological samples. In particular embodiments, the sample is a biological sample such as cells, cell extracts or lysates, tissues or tissue extracts, bodily fluids, serum, blood and blood products.

In some embodiments, the method of identifying or detecting a target molecule in a sample detects of identifies proteins, post-translationally modified forms of proteins, peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, ions, carbohydrates, polysaccharides, hormones, receptors, antigens, antibodies, viruses, metabolites, co-factors, drugs, dyes, nutrients, or growth factors. In other embodiments, the method of the invention detects of identifies proteins or post-translationally modified forms of proteins. In some embodiments, the target is a post-translationally modified protein, where the post-translation modifications is phosphorylation, prenylation, glycosylation, methionine removal, N-acetylation, acylation, acylation of cysteines, myristoylation, alkylation, ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycoslylation, deamination of glutamine

and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation, or lipidation.

In some embodiments, the target is a protein kinase. In particular embodiments, the target is a phosphorylated protein kinase.

5   In another aspect, the invention includes a diagnostic system for identifying or detecting a target molecule, where the diagnostic system includes a nucleic acid sensor molecule of the invention and a detector in communication with the nucleic acid sensor molecule, wherein the detector detects changes in the signal generated by the optical signal generating unit of the nucleic acid sensor. In some embodiments, the diagnostic system

10   further includes a processor for processing signals detected by the detector.

In another aspect, the invention includes a method of identifying or detecting a protein kinase in a sample by contacting a sample suspected of containing a protein kinase with a nucleic acid sensor molecule, wherein said nucleic acid sensor molecule has a target recognition domain that recognizes a protein kinase, and wherein a change in the signal

15   generated by the optical signal generating unit indicates the presence of protein kinase in the sample. In some embodiments, the method further includes quantifying the amount of signal generated by the optical signal generating unit to quantify the amount of protein kinase in the sample.

In yet another aspect, the invention provides a method of identifying a modulator of

20   protein kinase activity by contacting a test agent with a protein kinase and nucleic acid sensor molecule, wherein the nucleic acid sensor molecule has a target recognition domain that recognizes a protein kinase, recognition of the protein kinase by the target recognition domain of the nucleic acid sensor molecule results in a change in the signal generated by the optical signal generating unit and wherein changes in the signal generated by the optical

25   signal generating unit in the presence and absence of the test agent indicates the test agent is a modulator of the protein kinase activity.

In some embodiments, the catalytic domain of the nucleic acid sensor molecule includes a cis-ligase ribozyme or a trans-ligase ribozyme.

In another aspect, the invention provides a nucleic acid sensor molecule which

30   includes a target modulation domain that recognizes ERK, a catalytic domain that includes a ligase or cis-hammerhead, and a linker domain that links the target modulation domain and the catalytic domain.

In another aspect, the invention includes a nucleic acid sensor molecule which includes a target modulation domain that recognizes phosphoERK, a catalytic domain that

includes a ligase or a cis-hammerhead, and a linker domain that links the target modulation domain and the catalytic domain.

In yet another aspect, the invention provides a nucleic acid sensor molecule which includes a target modulation domain that recognizes lysozyme, a catalytic domain that

5   includes a 1-piece cis-ligase and a linker domain that links the target modulation domain and the catalytic domain.

In still another aspect, the invention provides a nucleic acid sensor molecule which includes a target modulation domain that recognizes any one of cCMP, cAMP, or cGMP, a catalytic domain, and a linker domain that links the target modulation domain and the

10   catalytic domain, wherein the nucleic acid sensor molecule includes an optical signal generating unit or a non-radioactive detectable label. In some embodiments, the nucleic acid sensor molecule includes an optical signal generating unit. In other embodiments, the nucleic acid sensor molecule includes a detectable label. In a particular embodiment, the label is a radioactive label, such as, for example, ^{32}P, ^{33}P, ^{14}C, ^{35}S, ^{3}H, or ^{125}I. In other

15   embodiments, the nucleic acid sensor molecule comprises a fluorescent label, such as, for example, fluorescein, DABCYL, or a green fluorescent protein (GFP) moiety. In some embodiments, the optical signal generating unit includes a fluorescent moiety and a quenching moiety, wherein recognition by the target modulation domain causes causes a change in detectable fluorescence by the optical signal generating unit. In some

20   embodiments, the nucleic acid sensor molecule includes an enzymatic label, or an affinity capture tag label.

In some embodiments, the nucleic acid sensor molecule includes a target modulation domain recognizes ERK1, ERK2 or both. In a particular embodiment, the nucleic acid sensor molecule includes a target modulation domain and catalytic domain are as shown by

25   SEQ ID NO. 80, and the linker is randomized.

In some embodiments, the nucleic acid sensor molecule includes a target modulation domain and a catalytic domain as shown in any one of SEQ ID NOS. 47, 118 and 119, and the linker is randomized.

In another aspect, the invention provides a nucleic acid sensor molecule that

30   recognizes ERK having the SEQ ID NO. 90-95, 108-116, 131-133, 140-295, 349, 351, or 356.

In another aspect, the invention provides a nucleic acid sensor molecule that recognizes phospoERK having the SEQ ID NO. 5-8, 37-39, 44-45, 81-89, 96-100, 121-130, 352, or 353.

In another aspect, the invention provides a nucleic acid sensor molecule that recognizes any one of cCMP, cAMP or cGMP, having the SEQ ID NO. 40-43, 103, or 135-139.

In another aspect, the invention provides a nucleic acid sensor molecule that recognizes lysozyme, having the SEQ ID NO. 46, 47, 76, or 105-107.

In another aspect, the invention provides a 1-piece ligase ribozyme including a target modulation domain that recognizes a target, a linker domain, and a catalytic domain wherein the 5' and 3' ends of the ligase ligate to each other upon recognition of the target by the modulation domain.

In another aspect, the invention provides a 2-piece ligase ribozyme including a target modulation domain that recognizes a target, a linker domain, and a catalytic domain including an oligonucleotide substrate ligation site and and oligonucleotide supersubstrate binding domain, wherein upon recognition of the target by the modulation domain, the 3' end of the an oligonucleotide supersubstrate is ligated to the 5' end of the oligonucleotide substrate ligation site.

In yet another aspect, the invention provides a 3-piece ligase ribozyme including a target modulation domain that recognizes a target, a linker domain, and a catalytic domain comprising comprising an oligonucleotide substrate binding domain capable of binding an oligonucleotide substrate and an effector-oligonucleotide binding site capable of binding an effector oligonucleotide, wherein upon recognition of the target by the modulation domain, and in the presence of binding of the effector oligonucleotide to the effector-oligonucleotide binding site, then the 3' end of the oligonucleotide substrate is ligated to the 5' end of the ligase.

The invention also provides a 1-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 47, 105-107, 119.

The invention also provides a 2-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 347, 349, and 351.

The invention further provides a 3-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 46, 75, 76, 108-116, 118, 121-130, and 352.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and

materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

5   Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

10   Figure 1 depicts methodology for selecting nucleic acid sensor molecules of the invention. Figure 1A is a flow diagram showing a method for selecting nucleic acid sensor precursor molecules having a target molecule activatable ligase activity according to one embodiment. Figure 1B is a flow diagram showing a gel-based method for selecting nucleic acid sensor precursor molecules having a target molecule activatable endonuclease activity

15   according to one embodiment.

       Figures 2A and B show a nucleic acid sensor molecule (SEQ ID NO:46) according to one embodiment, in which the catalytic site includes a ligase site. Figure 2A shows the conformation of the target molecule (SEQ ID NO:46) bound form of the nucleic acid sensor molecule with an effector oligo hybridized to its 3' end (SEQ ID NO:51). Figure 2B shows

20   the conformation of the non-target bound form of the nucleic acid (SEQ ID NO:46) sensor molecule.

       Figures 3A and B show a nucleic acid sensor molecule (SEQ ID NO:47) derived from the nucleic acid sensor precursor molecule shown in Figures 2A and B in which first and second nucleotides are labeled with first and second signaling moieties (F and D,

25   respectively).

       Figure 4 is a flow diagram showing a method for selecting nucleic acid sensor molecules having a target molecule activatable self-cleavage activity according to one embodiment.

       Figures 5A and B show a nucleic acid sensor molecule according to one

30   embodiment, in which the catalytic site includes a self-cleavage site which is the catalytic core of a hammerhead ribozyme. Figure 5A shows the conformation of the target molecule bound form of the nucleic acid sensor molecule (SEQ ID NO:48). Figure 5B shows the conformation of the non-target bound form of the nucleic acid sensor molecule (SEQ ID NO:48).

Figures 6A and B show a nucleic acid sensor molecule derived from the nucleic acid sensor molecule shown in Figures 3A and B in which first and second nucleotides are labeled with first and second signaling moieties (F and D, respectively) (SEQ ID NO:49).

Figure 7 is a schematic diagram illustrating pathway target molecules according to one embodiment.

Figure 8 is a flow chart showing steps in a drug optimization method according to one embodiment, in which nucleic acid sensor molecules are used at each step in the method.

Figure 9A shows an example of a self-cleaving nucleic acid sensor bound to a solid support when used in an epi-illuminated FRET detection scheme. Figure 9B shows the same sensor in an epi-illuminated beacon configuration, with the acceptor fluorophore replaced by a quencher group. Figure 9C shows the same sensor in an TIR-illuminated beacon configuration.

Figure 10A shows an example of a self-ligating nucleic acid sensor bound to a solid support when used in a TIR-illuminated detection scheme where there is a signal increase upon target binding. Figure 10B shows the same sensor in an epi-illuminated configuration, where target binding is detected by monitoring changes of the fluorophore bound to the substrate at the surface of the array. Figure 10C shows the same epi-illuminated configuration, where target binding is detected by monitoring changes in the fluorescence polarization.

Figure 11 illustrates the use of beads in a homogeneous assay format utilizing a self-ligating nucleic acid sensor. Figure 11A shows the beads prior to target binding and ligation (no emission from acceptor). Figure 11B shows the beads after target binding and ligation (emission from acceptor detected).

Figure 12 shows a schematic of a previously constructed scanning detection system utilizing TIR laser evanescent wave excitation in either large area illumination/CCD imaging mode, or scanned spot/PMT imaging mode. The schematic shows how an array can be scanned and FP or fluorescence intensity data extracted.

Figure 13. shows a schematic of fluorescence data generated by a biosensor array using the indicated nucleic acid sensors and target molecules.

Figures 14 shows a schematic showing different catalytic platforms for detection methods for nucleic acid sensor molecules.

Figure 15 is a schematic showing a ligase nucleic acid sensor molecule sensor system. It shows SEQ ID NO:76 across the top, and SEQ ID NO:77 hybridized to a portion of SEQ ID NO:76 and bound to an insulating moiety.

Figure 16 is a schematic showing a hammerhead (endonuclease) Nucleic acid sensor molecule sensor system. It has a nucleic acid sensor molecule sensor (SEQ ID NO:78), and SEQ ID NO:79 hybridizing to a portion of the sensor and bound to an insulating moiety.

Figure 17 is a schematic of the net electron transfer to or from the electrode.

Figure 18 is a schematic of a peak in the faradaic current, centered at the redox potential of the electron donor species (specified for a given reference electrode) and superposed on top of the capacitive current baseline which is observed in the absence of surface-immobilized signaling probes.

Figure 19 is the sequence of the entire ERK2 activated allosteric ribozyme (SEQ ID NO:80). Also shown are the sequences of the Stem II connector domain for selective clones.

Figure 20A is a chart showing measurement of cis-hammerhead cleavage. Figure 20B shows a chart showing measurement of cis-hammerhead cleavage.

Figure 20C is a chart showing measurement of cis-hammerhead cleavage.

Figure 21 is a chart showing ERK2-dependence of cis-hammerhead cleavage.

Figure 22A is a chart showing the measurement of ERK2-inhibitor IC50 values by nucleic acid sensor molecule.

Figure 22B is a chart showing the fraction of construct 1-14 cleaved in the presence of 100 nM ERK2, ricin, or MEK, or with no protein.

Figure 23A shows a ppERK cis-hammerhead nucleic acid sensor molecule construct (SEQ ID NO:353).

Figure 23B shows construct 6 (SEQ ID NO:81) 7 (SEQ ID NO:82), 8 (SEQ ID NO:83), 9 (SEQ ID NO:84), 10 (SEQ ID NO:85), 11 (SEQ ID NO:86) 12 (SEQ ID NO:87), 13 (SEQ ID NO:88), and 14 (SEQ ID NO:89).

Figure 24 shows the linker region, activity, and stability of constructs 6 through 14.

Figures 25A and B show a bar graph and corresponding radiograph demonstrating the relative pp ERK dependence of constructs 6, 10, and 12.

Figure 26 shows the sequences for lysozyme modulated ligase nucleic acid sensor molecules C.lys.L1.A (SEQ ID NO:105), C.lys.L1.B (SEQ ID NO:106), and C.lys.L1.C (SEQ ID NO:107).

Figure 27 is a schematic of how an L1 ligase is configured for self-circularization, and how its self-circularization can be detected using RT-PCR.

Figure 28 shows increases in amplification of circularized C.lys.L1.B (SEQ ID NO:106) in response to the addition of lysozyme. The signal is strengthened as additional
5   cycles of PCR are performed.

Figure 29 shows that the ligase nucleic acid sensor molecule C.lys.L1.B (SEQ ID NO:106) still self ligates in response to the presence of lysozyme, even in the presence of HeLa cell extract, demonstrating the stability of this nucleic acid sensor molecule.

Figure 30 shows modulation of a 1-piece ligase nucleic acid sensor molecule *in vivo*.
10   Figure 31 is a schematic showing the construct design for an ERK dependent 3-piece ligase nucleic acid sensor molecule (SEQ ID NO:118).

Figure 32 shows the sequences for constructs 17 (SEQ ID NO:109), 18 (SEQ ID NO:110), 19 (SEQ ID NO:111), 20 (SEQ ID NO:112), and 21 (SEQ ID NO:113), 22 (SEQ ID NO:114), 23 (SEQ ID NO:115), 24 (SEQ ID NO:116), 25 (SEQ ID NO:117), and 26
15   (SEQ ID NO:118).

Figure 33 shows the ERK dependent activity of constructs 17 (A), 18 (B), 20 (C), 21 (D), 19 (E), 22 (F), 23 (G), 25 (H), and 26 (I).

Figure 34 is a graph that shows ligase time-dependent activity assays for construct 17 (clone A) (SEQ ID NO:109) and construct 19 (clone E) (SEQ ID NO:111).
20   Figure 35 shows a graph showing the time-dependent activity of construct 19 (clone E) (SEQ ID NO:111) with varying concentrations of ERK2.

Figure 36 shows secondary structure representations of 3-piece construct 27 (SEQ ID NO:118) and 1-piece construct 28 (SEQ ID NO:119) ERK dependent ligases. 1-piece ERK dependent ligase is a slightly modified version of 3-piece system where the effector
25   and substrate regions are replaced by a stable GNRA tetraloop.

Figure 37 shows a graph demonstrating continued ERK2 dependence of a nucleic acid sensor molecule in the 3-piece and 1-piece formats (constructs 19 and 28, respectively).

Figure 38 shows a secondary structure representation of the 2-piece ERK dependent ligase platform (SEQ ID NO:347), and its oligonucleotide substrate (SEQ ID NO:350).
30   Figure 39A shows a secondary structure representation of two 2-piece ERK-modulated nucleic acid sensor molecules: construct 29 (SEQ ID NO:349), and construct 30 (SEQ ID NO:351), with their oligonucleotide substrate (SEQ ID NO:348).

Figure 39B shows ligation assays run on constructs 29 (SEQ ID NO:349) and 30 (SEQ ID NO:351), in the absence or in the presence of 1 uM ERK.

Figure 40 shows the ligation efficiency of ERK nucleic acid sensor molecule construct 19 (SEQ ID NO:111) detected with quantitiative-PCR (Taqman®). All incubation with various concentration of ERK were performed in the presence of 10% 293 extracts with exogenously added 10 mM MgCl$_2$.

5   Figure 41 shows a schematic of the template for ppERK dependent ligase nucleic acid sensor molecules (SEQ ID NO:352) and its oligonucleotide substrate (SEQ ID NO:350).

Figure 42 shows the nucleotide sequences for construct 31 (TK.16.118.K) (SEQ ID NO:121), construct 32 (TK.16.118.L) (SEQ ID NO:122), construct 33 (TK.16.118.M) (SEQ

10   ID NO:123), construct 34 (TK.16.118.N) (SEQ ID NO:124), construct 35 (TK.16.118.O) (SEQ ID NO:125), construct 36 (TK.16.118.P) (SEQ ID NO:126), construct 37 (TK.16.118.Q) (SEQ ID NO:127), construct 38 (TK.16.118.R) (SEQ ID NO:128), construct 39 (TK.16.118.S) (SEQ ID NO:129), and construct 40 (TK.16.118.T) (SEQ ID NO:130).

Figure 43 shows template sequences for the creation of a ppERK (SEQ ID NO:354)

15   or ERK (SEQ ID NO:355) library of nucleic acid sensor molecules.

Figure 44A shows the stem sequences of ERK dependent nucleic acid clones CW45-33-A08 (SEQ ID NO:356), CW45-33-C08 (SEQ ID NO:131), CW45-33-C09 (SEQ ID NO:132), CW45-33-D09 (SEQ ID NO:133), CW45-33-F08 (SEQ ID NO:90), CW45-33-H08 (SEQ ID NO:91), CW45-33-H09 (SEQ ID NO:92), CW45-33-A10 (SEQ ID NO:93),

20   CW45-33-F09 (SEQ ID NO:94), and CW45-33-G08 (SEQ ID NO:95).

Figure 44B shows the stem sequences of pp ERK dependent nucleic acid clones CW45-33-A02 (SEQ ID NO:44), CW45-33-B04 (SEQ ID NO:45), CW45-33-C04 (SEQ ID NO:5), CW45-33-D04 (SEQ ID NO:6), CW45-33-F03 (SEQ ID NO:7), CW45-33-D01 (SEQ ID NO:8), CW45-33-D02 (SEQ ID NO:37), CW45-33-D05 (SEQ ID NO:38), CW45-

25   33-E01 (SEQ ID NO:39), CW45-33-G02 (SEQ ID NO:96), CW45-33-G03 (SEQ ID NO:97), CW45-33-H03 (SEQ ID NO:98), CW45-33-H1 (SEQ ID NO:99), and CW45-33-B05 (SEQ ID NO:100).

Figure 45 nucleotide sequences of CW45-33-A02 (SEQ ID NO:44), and CW45-33-D04 (SEQ ID NO:6).

30   Figure 46 shows a schematic demonstrating amplicon-dependent nucleic acid amplification (ADNA).

Figure 47 shows a schematic describing a mechanism used by nucleic acid sensor molecules to transduce signal and the kinetic constants used to characterize NASMs.

Figure 48 shows a graph demonstrating the determination of threshold cycle versus log of target molecule concentration using amplicon-dependent nucleic acid amplification via quantitative PCR analysis.

Figure 49 shows a graph demonstrating the determination of threshold cycle versus log of target molecule concentration using amplicon-dependent nucleic acid amplification via SYBR-green analysis.

Figure 50 shows a radiograph demonstrating lysozyme sensitive ligase nucleic acid sensor molecule activity in reticulocyte and HeLa cell extract.

Figure 51 shows a radiograph showing that ligase activity is relatively unchanged in the presence of cell lysate and various RNase inhibitors.

Figure 52 shows a radiograph of a lysozyme modulated nucleic acid sensor molecule in the presence of human serum.

Figure 53 shows a schematic describing rolling circle amplification of an amplicon derived from immobilized trans-acting ligase nucleic acid sensor molecules.

Figure 54 shows a schematic describing exponential amplification of an amplicon.

Figure 55 shows a schematic describing FRET-based signal generation coupled to nucleic acid synthesis of nucleic acid sensor molecules.

Figure 56 shows a schematic describing cellular assays using 1-piece ligase nucleic acid sensor molecules.

Figure 57 shows bar graphs plotting the rate of activity of nucleic acid sensors when in the presence of different target molecules *in vitro*. Panel A shows the rate of activity in the presence of ERK and phosphorylated ERK for construct 19 (ligase E) on the left bar and construct 33 (ligase M) on the right bar. Panel B shows construct 19 and 33 rates of activity in the presence of Ras, MEK, ERK, p38, and ricin.

Figure 58 shows a graph describing the activity of an ERK modulated nucleic acid sensor molecule in the presence of 10% 293 cell extract in the left panel. The right panel shows the activity of an ERK modulated nucleic acid sensor molecule in the presence of increasing concentrations of staurosporine. Both panels show data determined using quantitive PCR methods (ADNA).

Figure 59 lists the switch factor, dissociation constants, catalytic constant and detection limit for an ERK aptamer in comparison to four ERK dependent ligase nucleic acid sensor molecules.

Figure 60A and B show RT-PCR gels, and C and D corresponding bar graphs showing ERK modulation of a nucleic acid sensor molecule *in vitro* (panels A and C) and in biological extracts (panels B and D).

Figure 61 shows a schematic describing optical detection based on the modulation of an intron-derived nucleic acid sensor molecule.

Figure 62 shows the original solution-phase cGMP-dependent hammerhead nucleic acid sensor molecule FRET construct (SEQ ID NO:101) and its effector/capture oligo (SEQ ID NO:102) from which the solid-phase FRET sensor was derived. In the solution-phase construct shown in Figure 62A, the fluorophore (F) and quencher (Q) are FAM and DABCYL, respectively. In the solid-phase construct shown in Figure 62B, the donor fluorophore (D) and acceptor fluorophore (A) are FAM and AlexaFluor 568, respectively.

Figure 63A shows the surface-immobilized FRET sensor before, and Figure 63B shows after, exposure to the activating target molecule (cGMP), followed by subsequent cleavage and dissociation of the sequence fragment containing the acceptor fluorophore (A). Figure 63C shows the expected kinetic time course signals and Figure 63D shows the actual kinetic time course signals observed from these sensors in the presence of various concentrations of target.

Figure 64 shows fitted kinetic time course signals observed from the solid-phase FRET sensor constructs in a solution-phase assay. Figure 64A shows a graph that plots the signal observed from the donor fluorophore only in the presence of 200 uM cGMP. Figure 64B shows a graph of the parametric fit to the experimental data shown in Figure 64A, verifying that the rate constant for the solid-phase construct is in fact similar to that for the solution-phase construct under similar conditions.

Figures 65A and 65B compare the observed pseudo-first order rate constants from solution- and solid-phase FRET sensor constructs. Figure 65 C, D, and E shows experimental data and constructs for multiplexed detection using solution-phase cGMP and cAMP FRET.

Figure 66A shows an endonuclease (hammerhead ribozyme)-based nucleic acid sensor immobilized linked to a gold surface via a thiol linker. Figure 66B shows the fraction of this type of sensor cleaved and dissociated as a function of time in the presence of a fixed concentration of target. Figure 66C shows the signal (image density) from a panel of immobilized sensors prior to their exposure to a target-mixture. Figure 66D shows the signal from the uncleaved sensors after exposure to the mixture of all listed targets, while

Figure 66E represents the target-dependent cleavage signal. Specific target-dependent activity of each sensor is seen in each case for this multiplexed assay.

Figure 67 shows a schematic diagram of the integrated SPReeta SPR sensor module (Figure 67A), as well as the nucleic acid (hammerhead ribozyme) sensor molecule that is immobilized on the gold SPR layer (Figure 67B). Figures 67C and 67D show typical real-time data generated by the SPR sensor system during sensor loading and target analyte-induced cleavage, respectively.

Figure 68 gives the sequences for three cyclic nucleotide-dependent nucleic acid (hammerhead ribozyme) sensors dependent upon cGMP (SEQ ID NO:135), cCMP (SEQ ID NO:136), and cAMP (SEQ ID NO:137) in a conformation suitable for direct 5' surface attachment. The schematic shows the SPR sensor construct intended for direct 5' attachment to a native gold surface via a terminal thiol linker.

Figure 69 gives the sequences for three cyclic nucleotide-dependent nucleic acid (hammerhead ribozyme) sensors dependent upon cCMP (SEQ ID NO:138), cAMP (SEQ ID NO:103), and cGMP (SEQ ID NO:139) in a conformation suitable for direct 3' surface attachment. The figure schematic shows the SPR sensor construct intended for direct 3' attachment to a neutravidin surface which has been passively adsorbed onto the gold SPR surface via cysteine residues.

Figure 70 gives the sequences for three cyclic nucleotide-dependent nucleic acid (hammerhead ribozyme) sensors dependent upon cCMP (SEQ ID NO:40), cAMP (SEQ ID NO:41), and cGMP (SEQ ID NO:42)in a conformation suitable for indirect surface attachment via a capture oligo. The schematic shows the SPR sensor construct intended for indirect surface attachment via a capture oligo to a neutravidin surface which has been passively adsorbed onto the gold SPR surface via cysteine residues.

Figure 71 shows the surface loading (Figure 71A) and target-dependent cleavage (Figure 71B) of cGMP-dependent nucleic acid (hammerhead ribozyme) sensor molecules, as well as the physical configurations of the various nucleic acid sensor molecules (Figure 71C) that give rise to the indicated portions of the kinetic data shown. The high signal to noise ratio (SNR) and dynamic range (DNR) of this SPR sensor array are clearly evident from the plots.

Figure 72 shows a plot of SPR signal (in refractive index units, RIU) vs. time for a typical SPR sensor array assay: surface cleaning (dH$_2$0, NaOH, PBS), surface loading of the gold SPR layer with neutravidin (NA), requilibration with PBS (PBS), loading of the

surface with biotinylated sensor molecules in PBS(HH+B/PBS), requilibration of sensor surface in assay buffer (HH buff), and addition of target in assay buffer (target/HH buff).

Figure 73 shows a schematic representation of the secondary structure and components of a ligase-based nucleic acid array sensor. The sensor (SEQ ID NO:75) is

5   shown attached to the chip surface via hybridization to a capture oligo (SEQ ID NO:104), and with an external substrate oligo bearing a fluorescent label already ligated into place. The substrate oligo can be either directly labeled (as shown), or labeled with an affinity tag (e.g., biotin) for subsequent indirect labeling or signal amplification (e.g., via tyramide signal amplification).

10   Figure 74 contrasts the two principal solid-phase array (chip) formats used for ligase-based nucleic acid sensors.

Figure 75 shows a multiplex in situ ligase sensor chip, with pre-immobilized radiolabeled sensors activatable by lysozyme (LYS) and FMN.

Figure 76 shows dose-response data for ERK-dependent ligase-based nucleic acid

15   sensors using a gel-assay (panel A) and a capture chip (pane lB).

Figure 77 shows dose-response data for an in situ ligase-based nucleic acid sensor array populated with ERK-dependent unlabeled ligase sensors in Figure 77A and in Figure 77B the retained ligation signal from each spot in the concentration profile was plotted vs. its corresponding target concentration.

20   Figure 78 shows dose-response data for a ERK-dependent ligase-based nucleic acid sensor capture array in Figure 78A and captured and amplified fluorescent ligation signal for each spot is plotted in Figure 78B vs. its corresponding target concentration.

Figure 79 shows the components of a generalized construct for an amplifiable ligase-based nucleic acid sensor molecule.

25   Figure 80 shows a generalized strategy for performing a multiplexed capture chip formatted assay with ligase-based nucleic acid sensors.

Figure 81 shows a generalized strategy for performing a highly sensitive capture chip formatted assay with ligase-based nucleic acid sensors.

Figure 82 shows a schematic describing multiplexed chip assays.

30

Detailed Description of the Invention

The invention is generally drawn to catalytic NASMs (also know as allosteric ribozymes, aptazymes and the like) and optical nucleic acid sensor molecules that may be used to monitor the presence or concentration of various target molecules. Target

molecules include a variety of biologically relevant molecules, such as, for example, proteins (including specific post-translationally modified forms of proteins), peptides, nucleic acids, nucleotides, natural products, metabolites, drugs, toxins, biohazards, and ions.

The invention also includes methods by which a change in the activity or conformation of a nucleic acid sensor molecule upon recognition of a specific target molecule can be coupled to a quantifiable, measurable signal. The invention also includes methods which allow one to test the inhibitory activity of one or more compounds simultaneously against one or more enzymes or biochemical targets. Assays can be carried out in a variety of formats, including *in vitro* biochemical assays, *in vitro* cellular assays, *in vivo* cellular assays, in solution, on chips or other substrates, or *in vivo* animal models. These assays have applications in all phases of drug discovery, including target validation and discovery and development, high throughput screening, biochemical assays, *in vitro* cellular models and *in vivo* animal models.

Nucleic acid sensor molecules are RNAs, DNAs, RNA/DNA hybrids, or derivatives or analogs of nucleic acids that catalyze a chemical reaction and/or undergo a conformational change upon the recognition of a specific target molecule. Nucleic acid sensor molecule - based assays can be carried out using all catalytic platforms, which include endonucleases, such as the hammerhead ribozyme, the hairpin ribozyme, the HDV ribozyme, and the VS ribozyme; ligases, such as the L1 ligase, and the class I-III ligases and; group I and group II self-splicing introns.

Catalytic NASMs can be generated or selected by a variety of methods both disclosed herein and known in the art. For examples, see WO98/27104, WO01/96559, and WO 00/26226

Also disclosed herein are optical nucleic acid sensor molecules and methods making them. In general, optical catalytic NASMs generate a detectable optical signal upon recognition of a target molecule. Optical NASMs are generated from catalytic NASMs by addition of an optical signal generating unit.

Also disclosed herein are two new classes of catalytic NASMs (referred to as one-piece or cis ligases) and two piece ligases and methods of generating them.

In general, catalytic NASMs can be used, *e.g.*, to detect target molecules either by generation of an optical signal or an amplicon detectable, *e.g.*, by RT-PCR, size gel purification procedures and any other means of seperating variously sized or conformed nucleic acid molecules. Optical NASMs, on the other hand, can be used, *e.g.*, to detect target molecules by generation of an optical signal.

Optical signals can be generated by optical NASMs in a number of ways. In some embodiments the signal is an optical signal generated, *e.g.*, by the fluorescence of a fluorescent dye. In other embodiments, the signal is an optical signal generated by molecules in close proximity to the nucleic acid sensor molecule whose optical or

5   electrochemical properties are affected by the presence of the target molecule bound nucleic acid sensor molecule. In some embodiments the nucleic acid sensor molecules comprise at least one signaling moiety. In other embodiments, the nucleic acid sensor molecules comprise first and second signaling moieties whose optical properties change in response to the binding of a target molecule through changes in the proximity of the first and second

10   signaling moieties. Thus, detection can be direct or indirect.

In one embodiment, a plurality of nucleic acid sensor molecules are provided, either in solution, or immobilized on a substrate, generating a biosensor. In a further embodiment, a diagnostic system is provided which comprises at least one biosensor in optical communication with a optical signal detector. Methods of using the diagnostic system are

15   also provided, as well as kits for performing the method.

In other embodiments, the NASMs are used to detect the presence of target molecules *in vivo*.

In order to more clearly and concisely describe and point out the subject matter of the claimed invention, the following definitions are provided for specific terms which are

20   used in the following written description and the appended claims.

As defined herein, a "**oligonucleotide**" is used interchangeably with the term "nucleic acid" and includes RNA or DNA (or RNA/DNA) sequences of more than one nucleotide in either single strand or double-stranded form. A "modified oligonucleotide" includes at least one residue with any of: an altered internucleotide linkage(s), altered

25   sugar(s), altered base(s), or combinations thereof.

As defined herein, a "**target molecule**" is any molecule to be detected. The term "target molecule" refers to, any molecule for which nucleic acid sensor molecule exists or can be generated and can be naturally occurring or artificially created.

As defined herein, a "**signature target molecule**" is a target molecule whose

30   expression is correlatable with a trait.

As used herein, a "**diagnostic signature target molecule**" is a signature target molecule whose expression is, by itself or in combination with other signature target molecules, diagnostic of a trait.

As used herein, a "pathway target molecule" is a target molecule involved in a biological or metabolic pathway and whose accumulation and/or activity is dependent on other target molecules in the same biological or metabolic pathway, or whose accumulation and/or activity affects the accumulation and/or activity of other target molecules in the same

5   biological or metabolic pathway.

As used herein, a "diagnostic pathway target molecule" is a pathway target molecule whose expression/activity and/or structural properties, by itself or in combination with other pathway target molecules, is diagnostic of a particular trait.

As used herein, a "profiling nucleic acid sensor molecule" is a nucleic acid sensor

10   molecule that recognizes a signature target molecule, a diagnostic signature target molecule, a pathway target molecule, and/or a diagnostic pathway target molecule.

As defined herein, a "biosensor" comprises a plurality of nucleic acid sensor molecules.

As defined herein, a "profiling biosensor" comprises a plurality of profiling nucleic

15   acid sensor molecules.

As used herein, a molecule which "naturally binds to DNA or RNA" is one which is found within a cell in an organism found in nature.

As defined herein, a "target modulation domain" is the portion of a nucleic acid sensor molecule which recognizes a target molecule. The target modulation domain is also

20   sometimes referred to herein as the "target activation site" or "effector modulation domain".

As used herein a "catalytic domain" is the portion of a nucleic acid sensor molecule possessing catalytic activity which is modulated in response to binding of a target molecule to the target modulation domain.

As used herein, a "linker region" or "linker domain" is a portion of a nucleic acid

25   sensor molecule by or at which the "target modulation domain" and "catalytic domain" are joined. Linker regions include, but are not limited to oligonucleotides of varying length, baseparring phosphodiester, phosphothiolate, and other covalent bonds, chemical moieties (e.g., PEG), PNA, formacetal, bismaleimide, disulfide, and other bifunctional linker reagents. The linker domain is also sometimes referred to herein as a "connector" or

30   "stem".

As used herein, a "random sequence" or a "randomized sequence" is a segment of a nucleic acid having one or more regions of fully or partially random sequences. A fully random sequence is a sequence in which there is an approximately equal probability of each base (A, T, C, and G) being present at each position in the sequence. In a partially random

sequence, instead of a 25% chance that an A, T, C, or G base is present at each position, there are unequal probabilities.

As defined herein, "**a fixed region**" is a nucleic acid sequence which is known.

As defined herein, "**amplifying**" means any step or process or any combination of steps or processes that increases the amount or number of copies of a molecule or class of molecules.

As defined herein, a "**catalytic nucleic acid sensor molecule**" is a nucleic acid molecule comprising a target modulation domain, a linker region, and a catalytic domain.

As defined herein, an 'optical nucleic acid sensor molecule" is a catalytic nucleic acid sensor molecule wherein the catalytic domain has been modified to emit an optical signal as a result of and/or in lieu of catalysis by the inclusion of an optical signal generating unit.

As defined herein, a "**nucleic acid sensor molecule**" or "**NASM**" refers to either or both of a catalytic nucleic acid sensor molecule and an optical nucleic acid sensor molecule.

As used herein, a "**signal**" is a detectable physical quantity, impulse or object.

As used herein, an "**optical signal**" is a signal the optical properties of which can be detected.

As defined herein, an "**optical signal generating unit**" is a portion of a nucleic acid sensor molecule comprising one or more nucleic acic sequences and/or non-nucleic acid molecular entities, which change optical or electrochemical properties or which change the optical or electrochemical properties of molecules in close proximity to them in response to a change in the conformation or the activity of the nucleic acid sensor molecule following recognition of a target molecule by the target modulation domain.

As defined herein, a nucleic acid sensor molecule which "**recognizes a target molecule**" is a nucleic acid molecule whose activity is modulated upon binding of a target molecule to the TMD a greater extent than it is by the binding of any non-target molecule or in the absence of the target molecule. The recognition event between the nucleic acid sensor molecule and the target molecule need not be permanent during the time in which the resulting allosteric modulation occurs. Thus, the recognition event can be transient with respect to the ensuing allosteric modulation (*e.g.*, conformational change) of the nucleic acid precursor molecule or nucleic acid sensor molecule.

As defined herein, an "**array**" or "**microarray**" refers to a biosensor comprising a plurality of nucleic acid sensor molecules immobilized on a substrate.

As defined herein, a "substrate" refers to any physical supporting surface, whether rigid, flexible, solid, porous, gel-based, or of any other material or composition.

As defined herein, an "amplicon" is the sequence of a nucleic acid sensor molecule with ligase activity covalently ligated to an oligonucleotide substrate.

As defined herein, a "cleavage substrate" is an oligonucleotide or portion of an oligonucleotide cleaved upon target molecule recognized by a target modulation domain of an endonucleolytic nucleic acid sensor molecule.

As defined herein, an "oligonucleotide substrate" is an oligonucleotide that is acted upon by the catalytic domain of a nucleic acid sensor molecule.

As defined herein, an "effector oligonucleotide" is an oligonucleotide that base pairs with the effector oligonucleotide binding domain of a nucleic acid sensor molecule with ligase activity.

As defined herein, an "effector oligonucleotide binding domain" is the portion of the nucleic acid sensor molecule with ligase activity which is complementary to the effector oligonucleotide.

As defined herein, a "capture oligonucleotide" is an oligonucleotide that is used to attach a nucleic acid sensor molecule to a substrate by complementarity and/or hybridization.

As defined herein, an "oligonucleotide substrate binding domain" is the portion on the nucleic acid sensor molecule with ligase activity that is complementary to and can base pair with an oligonucleotide substrate.

As defined herein, a "oligonucleotide supersubstrate" is an oligonucleotide substrate that is complementary to and can base pair with the oligonucleotide substrate binding domain and to the effector oligonucleotide binding domain of a nucleic acid sensor molecule with ligase activity. The oligonucleotide supersubstrate may or may not carry an affinity tag.

As defined herein, a "oligonucleotide supersubstrate binding domain" is the region of a nucleic acid sensor molecule with ligase activity that is complementary to and can base pair with the oligonucleotide supersubstrate.

As defined herein, "stem selection" refers to a process performed on a pool of nucleic molecules comprising a target modulation domain, a catalytic domain and an oligonucleotide linker region wherein the linker region is fully or partially randomized.

As defined herein, "rational design/engineering" refers to a technique used to construct nucleic acid sensor molecules in which a non-conserved region of a ribozyme is

replaced with a target modulation domain and joined to the catalytic domain of the ribozyme by an oligonucleotide linker region.

As defined herein, "amplicon dependent nucleic acid amplification" refers to a technique by which one can amplify the signal of a nucleic acid sensor molecule by use of
5   standard RT/PCR or Real-Time RT-PCR methods."

As defined herein, "switch factor" is the enhancement observed in the catalytic activity and/or catalytic initial rate of a nucleic acid sensor molecule upon recognition of a target molecule by the target modulation domain.

As defined herein, a "cis-ligase ribozyme" is a ligase ribozyme that ligates its 3'
10   end to its 5' end. The cis-ligase ribozyme is also referred herein as "1-piece ligase" and is a 1-component system where oligonucleotide substrate, oligonucleotide substrate binding domain, catalytic domain, effector oligonucleotide and effector oligonucleotide binding domains are fused in the format shown in Figure 39.

As defined herein, a "trans-ligase ribozyme" is a ligase ribozyme that ligates its 5'
15   end to the 3' end of an oligonucleotide substrate.

As defined herein, a "2-piece ligase" is a 2-component trans-ligase ribozyme. The first component consists of the catalytic domain, the linker region, the target modulation domain, the substrate binding domain and the effector oligonucleotide binding domain. The second component is the oligonucleotide substrate that is complementary to the substrate
20   binding domain and the effector oligonucleotide binding domain. This system follows the format shown in Figure 41.

As defined herein, a "3-piece ligase" is a 3-component trans-ligase ribozyme. The first component consists of the catalytic domain, the linker, the target modulation domain, the substrate binding domain and the effector oligonucleotide binding domain. The second
25   component is the effector oligonucleotide that is complementary to the effector oligonucleotide binding domain. The third component is the oligonucleotide substrate that is complementary to the substrate binding domain. This system follows the format of the 3-piece ligase platform shown in Figure 39.

1.   Generating a Target Specific Nucleic Acid Sensor Molecule
30

Catalytic nucleic acid sensor molecules (NASMs) are selected which have a target molecule-sensitive catalytic activity (e.g., ligation or self-cleavage) from a pool of randomized oligonucleotides. The catalytic NASMs have a target modilation domain to which the target molecule specifically binds and a catalytic domain for mediating a catalytic

reaction. Binding of a target molecule to the target modulation domain triggers a conformation change and/or change in catalytic activity in the nucleic acid sensor molecule. In one embodiment, by modifying (*e.g.*, removing) at least a portion of the catalytic domain and coupling it to an optical signal generating unit, an optical nucleic acid sensor molecule

5   is generated whose optical properties change upon binding of a target molecule to the target modulation domain. In one embodiment, the pool of randomized oligonucleotides comprises the catalytic site of a ribozyme.

A.   Selection and Generation of Catalytic Nucleic Acid Sensor Molecules

10   In one embodiment, a heterogeneous population of oligonucleotide molecules comprising randomized sequences is screened to identify a nucleic acid sensor molecule having a catalytic activity which is modified (*e.g.*, activated) upon interaction with a target molecule. Each oligonucleotide in the population comprises a random sequence and at least one fixed sequence at its 5' and/or 3' end. In one embodiment, the fixed sequence comprises

15   at least a portion of a catalytic site. In the embodiments shown in Figures 1 and 4, the random sequence is flanked at both ends with fixed sequences.

        In one embodiment, the random sequence portion of the oligonucleotide is about 15-70 (*e.g.*, 30-40) nucleotides in length and can comprise ribonucleotides and/or deoxyribonucleotides. Random oligonucleotides can be synthesized from phosphodiester-

20   linked nucleotides using solid phase oligonucleotide synthesis techniques well known in the art (*see, e.g.*, Froehler, et al., 1986a; 1986b. Oligonucleotides can also be synthesized using solution phase methods such as triester synthesis methods (*see, e.g.*, Sood, et al., 1977, and Hirose, et al., 1978). Typical syntheses carried out on automated DNA synthesis equipment yield 10^{15}-10^{17} molecules. Sufficiently large regions of random sequence in the sequence

25   design increases the likelihood that each synthesized molecule is likely to represent a unique sequence.

        To synthesize randomized sequences, mixtures of all four nucleotides are added at each nucleotide addition step during the synthesis process, allowing for random incorporation of nucleotides. In one embodiment, random oligonucleotides comprise

30   entirely random sequences; however, in other embodiments, random oligonucleotide can comprise stretches of nonrandom or partially random sequences. Partially random sequences can be created by adding the four nucleotides in different molar ratios at each addition step.

To generate oligonucleotide populations which are resistant to nucleases and hydrolysis, modified oligonucleotides can be used and can include one or more substitute internucleotide linkages, altered sugars, altered bases, or combinations thereof. In one embodiment, oligonucleotides are provided in which the P(O)O group is replaced by P(O)S

5   ("thioate"), P(S)S ("dithioate"), P(O)NR$_2$ ("amidate"), P(O)R, P(O)OR', CO or CH$_2$ ("formacetal") or 3'-amine (-NH-CH$_2$-CH$_2$-), wherein each R or R' is independently H or substituted or unsubstituted alkyl. Linkage groups can be attached to adjacent nucleotide through an -O-N-, or -S- linkage. Not all linkages in the oligonucleotide are required to be identical.

10   In further embodiments, the oligonucleotides comprise modified sugar groups, for example, one or more of the hydroxyl groups is replaced with halogen, aliphatic groups, or functionalized as ethers or amines. In one embodiment, the 2'-position of the furanose residue is substituted by any of an O-methyl, O-alkyl, O-allyl, S-alkyl, S-allyl, or halo group. Methods of synthesis of 2'-modified sugars are described in Sproat, et al., 1991;

15   Cotten, et al., 1991; and Hobbs, et al., 1973. The use of 2-fluoro-ribonucleotide oligomer molecules can increase the sensitivity of a nucleic acid sensor molecule for a target molecule by ten-to one hundred-fold over those generated using unsubstituted ribo- or deoxyribooligonucleotides (Pagratis, et al., 1997), providing additional binding interactions with a target molecule and increasing the stability of the nucleic acid sensor molecule's

20   secondary structure(s) (Kraus, et al., 1998; Pieken, et al., 1991; Lin, et al., 1994; Jellinek, et al. 1995; Pagratis, et al., 1997).

In the embodiments shown in Figures 1 and 4, the random sequence portion of the oligonucleotide is flanked by at least one fixed sequence which comprises a sequence shared by all the molecules of the oligonucleotide population. Fixed sequences include

25   sequences such as hybridization sites for PCR primers, promoter sequences for RNA polymerases (e.g., T3, T4, T7, SP6, and the like), restriction sites, or homopolymeric sequences, such as poly A or poly T tracts, catalytic cores (described further below), sites for selective binding to affinity columns, and other sequences to facilitate cloning and/or sequencing of an oligonucleotide of interest. In one embodiment, the fixed sequence is

30   approximately 50 nucleotides in length.

In a preferred embodiment, the fixed sequence comprises at least a portion of a catalytic site of an oligonucleotide molecule (e.g., a ribozyme) capable of catalyzing a chemical reaction. Catalytic sites are well known in the art and include, e.g. a ligase site (see Figure 2), the catalytic sites of Group I or Group II introns (see, e.g., U.S. Patent

Number 5,780,272), the catalytic core of a hammerhead ribozyme (*see, e.g.*, U.S. Patent

Number 5,767,263 and U.S. Patent Number 5,700,923, and Figure 5, herein) or a hairpin

ribozyme (*see, e.g.*, U.S. Patent Number 5, 631,359. Other catalytic sites are disclosed in

U.S. Patent Number 6,063,566, Koizumi et al., FEBS Lett. 239: 285-288 (1988), Haseloff

5   and Gerlach, Nature 334: 585-59 (1988), Hampel and Tritz, Biochemistry 28: 4929-4933

(1989), Uhlenbeck, Nature, 328: 596-600 (1987), and Fedor and Uhlenbeck, Proc. Natl.

Acad. Sci. USA 87: 1668-1672 (1990)).

Nucleic acid sensor molecules are generally selected in a 5 to 20 cycle procedure. In

one embodiment, heterogeneity is introduced only in the initial selection stages and does not

10   occur throughout the replicating process. Figure 1A shows a schematic diagram in which

the oligonucleotide population is screened for a nucleic acid sensor molecule which

comprises a target molecule activatable ligase activity. Figure 1B shows the hammerhead

nucleic acid sensor molecule selection methodology. Each of these methods are readily

modified for the selection of NASMs with other catalytic activities.

15   In the embodiment shown in Figure 1A, the ligation reaction involves covalent

attachment of an oligonucleotide substrate to the 5'-end of the NASM through formation of

a phosphodiester linkage. Other ligation chemistries can form the basis for selection of

NASMs (*e.g.*, oligonucleotide ligation to the 3'-end, alkylation's (Wilson & Szostak),

peptide bond formation (Zhang & Czech), Diels-Alder reactions to couple alkenes and

20   dienes (Seelig & Jaschke), etc.). For some chemistries, the chemical functional groups that

constitute the reactants in the ligation reaction may not naturally appear within nucleic

acids. Thus, it may be necessary to synthesize an RNA pool in which one of the ligation

reactants is covalently attached to each member of the pool (*e.g.*, attaching a primary amine

to the 5'-end of an RNA to enable selection for peptide bond formation).

25   In this embodiment, the oligonucleotide population from which the NASMs will be

selected is initially screened in a negative selection procedure to eliminate any molecules

which have ligase activity even in the absence of target molecule binding. A solution of

oligonucleotides (*e.g.*, 100 pM) comprising a 5' and 3' fixed sequence ("5'-fixed: random:

3'-fixed") is denatured with a 3' primer sequence ("3' prime") (*e.g.*, 200 pM) which binds

30   to at least a portion of the 3' fixed sequence. In one embodiment, the 5'-fixed:random:3'-

fixed sequence is 5'.

GGACUUCGGUCCAGUGCUCGUGCACUAGGCCGUUCGACC-N_{30-50}

CUUAGACAGGAGGUUAGGUGCCUCGUGAUGUCCAGUCGC-3' (SEQ ID NO:1),
where N represents a random sequence having 30 to 50 nucleotides and the 3' primer
sequence used is 5'-GCGACTGGACATCACGAG-3' (SEQ ID NO:2).

Ligation buffer (e.g., 30 mM Tris HCl, pH 7.4, 600 mM NaCl, 1 mM EDTA, 1%
NP-40, 60 mM MgCl₂) and a tagged oligonucleotide substrate sequence ("tag-substrate")
(e.g., Tag-UGCCACU) are added and the mixture is incubated for about 16 to about 24
hours at 25 °C in the absence of target molecule (STEP 1). Tags encompassed within the
scope include, e.g., radioactive labels, fluorescent labels, a chemically reactive species such
as thiophosphate, the first member of a binding pair comprising a first and second binding
member, each member bindable to the other (e.g., biotin, an antigen recognized by an
antibody, or a tag nucleic acid sequence). The reaction is stopped by the addition of EDTA.
Alternatively, the reaction can be terminated by removal of the substrate or addition of
denaturants (e.g. urea, formamide).

Ligated molecules are removed from pool of selectable molecules (STEP 2),
generating a population of oligonucleotides substantially free of ligated molecules (as
measured by absence of the tag sequence in the solution). In the embodiment shown in
Figure 1A, the tag is the first member of a binding pair (e.g., biotin) and the ligated
molecules ("biotin-oligonucleotide substrate:5'-fixed:random:3'-fixed") are physically
removed from the solution by contacting the sample to a solid support to which the second
member of the binding pair is bound ("S") (e.g., streptavidin). The eluant collected
comprises a population of oligonucleotides enriched for non-ligated molecules (5'-
fixed:random:3'-fixed). This step can be repeated multiple times until the oligonucleotide
population is substantially free of molecules having target-insensitive ligase activity.

This step allows for suppression of the ability of constitutively active molecules to
be carried through to the next cycle of selection. Physical separation of ligated and
unligated molecules is one mechanism by which this can be achieved. Alternatively, the
negative selection step can be configured such that catalysis converts active molecules to a
form that blocks their ability to be either retained during the subsequent positive selection
step or to be amplified for the next cycle of selection. For example, the oligonucleotide
substrate used for ligation in the negative selection step can be synthesized without a
capture tag. Target-independent ligases covalently self-attach the untagged oligonucleotide
substrate during the negative selection step and are then unable to accept a tagged form of
the oligonucleotide substrate provided during the positive selection step that follows. In
another embodiment, the oligonucleotide substrate provided during the negative selection

step has a different sequence from that provided during the positive selection step. When PCR is carried out using a primer complementary to the positive selection oligonucleotide substrate, only target-activated ligases will be capable of amplification. .

A positive selection phase follows. In this phase, more 3' primer and tagged
5 oligonucleotide substrate are added to the pool resulting from the negative selection step. Target molecules are then added to form a reacted solution and the reacted solution is incubated at 25 °C for about 2 hours (STEP 3). Target molecules encompassed within the scope include, e.g., proteins or portions thereof (e.g., receptors, antigen, antibodies, enzymes, growth factors), peptides, enzyme inhibitors, hormones, carbohydrates,
10 polysaccharides, glycoproteins, lipids, phospholipids, metabolites, metal ions, cofactors, inhibitors, drugs, dyes, vitamins, nucleic acids, membrane structures, receptors, organelles, and viruses. Target molecules can be free in solution or can be part of a larger cellular structure (e.g., such as a receptor embedded in a cell membrane). In one embodiment, a target molecule is one which does not naturally bind to nucleic acids.

15 In one embodiment, nucleic acid sensor molecules are selected which are activated by target molecules comprising molecules having an identified biological activity (e.g., a known enzymatic activity, receptor activity, or a known structural role); however, in another embodiment, the biological activity of at least one of the target molecules is unknown (e.g., the target molecule is a polypeptide expressed from the open reading frame of an EST
20 sequence, or is an uncharacterized polypeptide synthesized based on a predicted open reading frame, or is a purified or semi-purified protein whose function is unknown).

Although in one embodiment the target molecule does not naturally bind to nucleic acids, in another embodiment, the target molecule does bind in a sequence specific or non-specific manner to a nucleic acid sensor molecule. In a further embodiment, a plurality of
25 target molecules binds to the nucleic acid sensor molecule. Selection for NASMs specifically responsive to a plurality of target molecules (i.e. not activated by single targets within the plurality) may be achieved by including at least two negative selection steps in which subsets of the target molecules are provided.

In still a further embodiment, nucleic acid sensor molecules are selected which bind
30 specifically to a modified target molecule but which do not bind to non-modified target molecules. Targeted modifications include, e.g., post-translational modifications of a protein, such as phosphorylation, ribosylation, methylation (Arg, Asp, N, S, or O-directed), prenylation (e.g., farnesyl, geranylgeranyl, and the like), acetylation, acylation, allelic variations within a protein (e.g., single amino acid changes in a protein) and cleavage sites

in a protein. In another embodiment, intermediates in a chemical synthesis pathway can be targeted, as well as starting and final products. In still a further embodiment, stereochemically distinct species of a molecules can be targeted.

The reacted solution is enriched for ligated molecules (biotin-oligonucleotide substrate: 5'- fixed :random:3'-fixed) by removing non-tagged molecules (5'-fixed:random:3'-fixed) from the solution. For example, in one embodiment, the tagged oligonucleotide substrate comprises a biotin tag and ligated molecules are isolated by passing the reacted solution over a solid support to which streptavidin (S) is bound (STEP 4). Eluant containing non-bound, non-ligated molecules (5'-fixed:random:3'-fixed) is discarded and bound, ligated molecules (biotin-oligonucleotide substrate: 5'-fixed:random:3'-fixed) are identified as nucleic acid sensor molecules and released from the support by disrupting the binding pair interaction which enabled capture of the catalytically active molecules. For example, heating to 95° C in the presence of 10 mM biotin allows release of biotin-tagged catalysts from an immobilized streptavidin support. In another embodiment, the captured catalysts remain attached to a solid support and are directly amplified (described below) while immobilized. Multiple positive selection phases can be performed (STEPS 3 and 4). In one embodiment, the stringency of each positive selection phase is increased by decreasing the incubation time by one half.

Physically removing inactive species from the pool adds stringency to the selection process. However, to the extent that the ligation reaction increases the amplification potential of the NASMs, this step may be omitted. In the illustrated embodiment, for example, ligation of an oligonucleotide to the active species provides a primer binding site that enables subsequent PCR amplification using an oligonucleotide substrate complementary to the original oligonucleotide substrate. Unligated species do not necessarily need to be physically separated from other species because they are less likely to amplify in the absence of a covalently tethered primer binding site. Selected nucleic acid sensor molecules are amplified (or in the case of RNA molecules, first reverse transcribed, then amplified) using an oligonucleotide substrate primer ("S primer") (e.g., 5 '-AAAAAATGCACTGGACT-3' (SEQ ID NO:3)) which specifically binds to the litgated oligonucleotide substrate sequence (STEP 5). In one embodiment, amplified molecules are further amplified with a nested PCR primer that regenerates a T7 promoter ("T7 Primer") from the 5' fixed and the litigated oligonucleotide substrate sequence (STEP 6). Following transcription with T7 RNA polymerase (STEP 7), the oligonucleotide pool may be further selected and amplified to eliminate any remaining unligated sequences (5'-fixed:random:3'-

fixed) by repeating STEPS 3-7. It should be obvious to those of skill in the art that in addition to PCR, and RT-PCR, any number of amplification methods can be used (either enzymatic, chemical, or replication-based, *e.g.*, such as by cloning), either singly, or in combination. Exemplary amplification methods are disclosed in Saiki, et al., 1985; Saiki, et al., 1988; Kwoh, et al., 1989; Joyce, 1989; and Guatelli, et al., 1990.

Because the 3' primer (3' prime) (see STEP 3 in Figure 1A) is included in the ligation mixture, selected nucleic acid sensor molecules may require this sequence for activation. In cases where this is undesirable, the 3' primer may be omitted from the mix. Alternatively, the final nucleic acid sensor molecule can be modified by attaching the 3' primer via a short sequence loop or a chemical linker to the 3' end of the nucleic acid sensor molecule, thereby eliminating the requirement for added primer, allowing 3' primer sequence to self-prime the molecule.

As shown in Figure 1B, selection of a nucleic acid sensor molecule begins with the synthesis of a ribozyme sequence on a DNA synthesizer. Random nucleotides are incorporated generating pools of roughly 10^{16} molecules. Most molecules in this pool are non-functional, but a handful will respond to a given target and be useful as nucleic acid sensor molecules. Sorting among the billions of species to find the desired molecules starts from the complex sequence pool. Nucleic acid sensor molecule are isolated by an iterative process: in addition to the target-activated ribozymes that one desires, the starting pool is usually dominated by either constitutively active or completely inactive ribozymes. The selection process removes both types of contaminants. In the following amplification stage, thousands of copies of the surviving sequences are generated to enable the next round of selection. During amplification, random mutations can be introduced into the copied molecules — this 'genetic noise' allows functional NASMs to continuously evolve and become even better adapted as target-activated molecules. The entire experiment reduces the pool complexity from 10^{16} down to < 100.

Pool preparation. The starting library of DNA sequences is generated by automated chemical synthesis on a DNA synthesizer. This library of sequences is transcribed *in vitro* into RNA using T7 RNA polymerase and subsequently purified.

Negative selection incubation. In the absence of the desired target molecule of interest, the RNA library is incubated together with the binding buffer alone. During this incubation, non-allosteric (or non-target activated) ribozymes are expected to undergo cleavage.

Size-based Purification. Undesired members of the hammerhead pool, those that are constitutively active in the absence of the target molecule, are removed from the unreacted members by PAGE-chromatography; 7M Urea, 8% acrylamide, 1X TBE. Higher molecular weight species are eluted as a single broad band from the gel matrix into TBE

5   buffer, then purified for subsequent steps in the selection cycle.

Positive selection incubation. The remaining RNA pool is then incubated under identical conditions but now in the presence of the target molecule of interest in binding buffer.

Size-based Purification. In this step, desired members of the hammerhead pool,

10   those that are only active in the presence of the target molecule, are removed from the remaining unreacted members by PAGE-chromatography; 7M Urea, 8% acrylamide, 1X TBE. In this step, lower molecular weight species are eluted as a single broad band from the gel matrix into TBE buffer, then purified for subsequent steps in the selection cycle.

Amplification. RT-PCR amplified DNA is purified and transcribed to yield an

15   enriched pool for a subsequent round of reselection.

Iteratively repeat. Rounds of selection and amplification (steps 2-5) are repeated until functional members sufficiently dominate the resultant library.

In another embodiment, as shown in Figure 4, an oligonucleotide population is screened for a nucleic acid sensor molecule which comprises a target molecule having

20   activatable self-cleaving activity. In this embodiment, the starting population of oligonucleotide molecules comprises 5' and 3' fixed regions ("5'-fixed and 3' fixed A-3'fixed B") and at least one of the fixed regions, in this example, the 3' fixed region, comprises a ribozyme catalytic core including a self cleavage site (the junction between 3' fixed A-3 'fixed B). In one embodiment, the 5'-fixed: random:3' fixed A-3'-fixed B

25   molecule is GGGCGACCCUGAUGAGCCUGG-N_{20-50}-UUAGACGAAACGGUGAAAGCCGUAGGUUGCCC (SEQ ID NO:4), where N_{20-50} is a random sequence of 20-50 nucleotides.

The population of oligonucleotide molecules comprising random oligonucleotides flanked by fixed 5' and 3' sequences (5'-fixed:random:3'-fixed A: 3' fixed B) are

30   negatively selected to remove oligonucleotides which self-cleave (i.e., 5'-fixed:random:3'-fixed-A molecules) even in the absence of target molecules. The oligonucleotide pool is incubated in reaction buffer (e.g., 50 mM Tris HCl, pH 7.5, 20 mM $MgCl_2$) for 5 hours at 25 °C, punctuated at one hour intervals by incubation at 60 °C for one minutes (STEP 1). In one embodiment, the uncleaved fraction of the oligonucleotide population (containing 5'-

fixed and 3' fixed A-3'-fixed B molecules) is purified by denaturing 10% polyacrylamide gel electrophoresis (PAGE) (STEP 2). Target molecule dependent cleavage activity is then selected in the presence of target molecules in the presence of reaction buffer by incubation at 23 °C for about 30 seconds to about five minutes (STEP 3). Cleaved molecules (5'-

5   fixed:random:3'fixed-A molecules) are identified as nucleic acid sensor molecules and are purified by PAGE (STEP 4).

Amplification of the cleaved molecule is performed using primers which specifically bind the 5'-fixed and the 3'-fixed A sequences, regenerating the T7 promoter and the 3'-   '
fixed B site (STEP 5), and the molecule is further amplified further by RNA transcription

10   using T7 polymerase (STEP 6). In one embodiment, the process (STEPS 1-6) is repeated until the nucleic acid sensor precursor population is reduced to about one to five unique sequences.

Alternative methods for separating cleaved from uncleaved RNAs can be used. Tags can be attached to the 3'-fixed B sequence and separation can be based upon

15   separating tagged sequences from non-tagged sequences at STEP 4. Chromatographic procedures that separate molecules on the basis of size (*e.g.*, gel filtration) can be used in place of electrophoresis. One end of each molecule in the RNA pool can be attached to a solid support and catalytically active molecules isolated upon release from the support as a result of cleavage. Alternate catalytic cores may be used. These alternate catalytic cores

20   and methods using these cores are also are encompassed within the scope of the invention.

Nucleic acid sensor molecules which combine both cleavage and ligase activities in a single molecule can be isolated by using one or a combination of both of the selection strategies outlined independently above for ligases and endonucleases. For example, the hairpin ribozyme is known to catalyze cleavage followed by ligation of a second

25   oligonucleotide substrate (Berzal-Herranz et al.). Target activated sensor precursors based on the hairpin activity can be isolated from a pool of randomized sequence RNAs prepared as described previously with a sequence of the form 5'-
GGAGTTACCTAACAAGAAACAGNgaagtcaaccagNgaaacNCACGGAGACGTGNNaNat taNctggt(N$_{20}$-

30   N$_{50}$)GGACCTACTGAGCTGACAGTCCTGTTTGATGCATACCGAGTAAGTG-3' (SEQ ID NO:36) where N indicates any nucleotide, lower case letters represent doped nucleotides, and uppercase letters represent fixed nucleotides. Hairpin-based NASMs can be isolated on the basis of target molecule dependent release of the fragment 5'-
GUCCUGUUUGAUGCAUACCGAGUAAGUG-3' (SEQ ID NO:74) in the same way that

hammerhead-based NASMs are isolated (*e.g.,* target molecule dependent increase in electrophoretic mobility or target molecule dependent release from a solid support). Alternatively, nucleic acid sensor molecules can be selected on the basis of their ability to substitute the 3'-sequence released upon cleavage for another sequence as described in an

5 target molecule independent manner by Berzal-Heranz et al. In this scheme, the original 3'-end of the NASM is released in an initial cleavage event and an exogenously provided oligonucleotide substrate with a free 5'-hydroxyl is ligated back on. The newly attached 3'-end provides a primer binding site that can form the basis for preferential amplification of catalytically active molecules. Constitutively active molecules that are not activated by a

10 provided target molecule can be removed from the pool by (1) separating away molecules that exhibit increased electrophoretic mobility in the absence of an exogenous oligonucleotide substrate or in the absence of target molecule, or (2) capturing molecules that acquire an exogenous oligonucleotide substrate (*e.g.,* using a 3'-biotinylated substrate and captured re-ligated species on an avidin column.

15 Like the hairpin ribozyme, the group I intron self-splicing ribozymes combine cleavage and ligation activities to promote ligation of the exons that flank it. In the first step of group I intron-catalyzed splicing, an exogenous guanosine cofactor attacks the 5'-splice site. As a result of an intron-mediated phosphodiester exchange reaction, the 5'-exon is released coincident with attachment of the guanosine cofactor to the ribozyme. In a

20 second chemical step, the 3'-hydroxyl at the end of the 5'-exon attacks the phosphodiester linkage between the intron and the 3'-exon, leading to ligation of the two exons and release of the intron. Group I intron-derived NASMs can be isolated from degenerate sequence pools by selecting molecules on the basis of either one or both chemical steps, operating in either a forward or reverse direction. NASMs can be isolated by specifically enriching

25 those molecules that fail to promote catalysis in the absence of target molecule but which are catalytically active in its presence. Specific examples of selection schemes follow. In each case, a pool of RNAs related in sequence to a representative group I intron (*e.g.,* the Tetrahymena thermophila pre-rRNA intron or the phage T4 td intron) serves as the starting point for selection. Random sequence regions can be embedded within the intron at sites

30 known to be important for proper folding and activity (*e.g.,* substituting the P5abc domain of the Tetrahymena intron, Williams et al.). Intron nucleic acid sensor molecules, in this case, sensitive to thio-GMP can be generated as follows.

First step, forward direction

The intron is synthesized with a short 5'-exon. In the negative selection step, a guanosine cofactor is provided and constitutively active molecules undergo splicing. In the positive selection step, the target molecule is provided together with thio-GMP. Molecules responsive to the target undergo activated splicing and as a result acquire a unique

5   thiophosphate at their 5'-termini. Thio-tagged NASMs can be separated from untagged ribozymes by their specific retention on mercury gels or activated thiol agarose columns.

First step, reverse direction

The method is performed as described in Green & Szostak. An intron is synthesized with a 5'-guanosine and no 5'-exon. An oligonucleotide substrate complementary to the 5'-

10   internal guide sequence is provided during the negative selection step and constitutively active molecules ligate the substrate to their 5'-ends, releasing the original terminal guanosine. A second oligonucleotide substrate with a different 5'-sequence is provided together with target in the positive selection step. NASMs specifically activated by the target molecule ligate the second oligonucleotide substrate to their 5'-ends. PCR

15   amplification using a primer corresponding to the second substrate can be carried out to preferentially amplify target molecule sensitive nucleic acid sensor molecules.

Second step, reverse direction

The method is performed as described in Robertson & Joyce. The intron is synthesized with no flanking exons. During the negative selection step, pool RNAs are

20   incubated together with a short oligonucleotide substrate under conditions which allow catalysis to proceed. During the positive selection step, a second oligonucleotide substrate with a different 3'-sequence is provided together with the sensor target. NASMs are activated and catalyze ligation of the 3'-end of the second substrate. Reverse transcription carried out using a primer complementary to the 3'-end of the second substrate specifically

25   selects NASMs for subsequent amplification.

Once nucleic acid sensor molecules are identified, they can be isolated, cloned, sequenced, and/or resynthesized using natural or modified nucleotides. Accordingly, synthesis intermediates of nucleic acid sensor molecules are also encompassed within the scope, as are replicatable sequences (*e.g.*, plasmids) comprising nucleic acid sensor

30   precursor molecules and nucleic acid sensor molecules.

> **B.**   **Converting a catalytic nucleic acid sensor molecule to an**

optical nucleic acid sensor molecule

The nucleic acid sensor molecules identified above through *in vitro* selection comprise a catalytic domain (i.e., a signal generating moiety), coupled to a target

5 modulation domain, (i.e., a domain which recognizes a target molecule and which transduces that molecular recognition event into the generation of a detectable signal). In general, the target modulation domain is defined by the minimum number of nucleotides sufficient to create a three-dimensional structure which recognizes a target molecule. In addition, the nucleic acid sensor molecules of the present invention use the energy of

10 molecular recognition to modulate the catalytic or conformational properties of the nucleic acid sensor molecule. The selection process as described in detail in the present invention identifies novel nucleic acid sensor molecules through target modulation of the catalytic core of a ribozyme. Hence, the *in vitro* selection procedures described herein are distinct from those previously described for affinity-based aptamer selections (e.g., SELEX) in that

15 we show that selective pressure on the starting population of NASMs (starting pool size is as high as 10^{14} to 10^{17} molecules) results in nucleic acid sensor molecules with enhanced catalytic properties, but not in enhanced binding properties, Figure 59. Specifically, the NASM selection procedures place selective pressure on catalytic effectiveness of potential NASMS by modulating both target concentration and reaction time-dependence. Either

20 parameter, when optimized throughout the selection, can lead to nucleic acid molecular sensor molecules which have custom-designed catalytic properties, e.g., NASMs that have high switch factors (Figure 47 and Figure 59), and or NASMs that have high specificity (Figure 57). The kinetic properties of the NASMs of the present invention are consistent with that obtained from a nucleoprotein-selection reported previously by Robertson and

25 Ellington (2001) in which the resulting ribozyme (switch factor equal to 1,700 fold) has the same affinity for RNA (1 μM) as did the starting pool.

In the examples described above, the catalytic site is a known sequence (a ligase site or a hammerhead catalytic core) and is at least a portion of either the 5' and/or 3' fixed region (the other portion being supplied by the random sequence), or is a complete catalytic

30 site. However, in other embodiments, the catalytic site may be selected along with the target molecule binding activity of oligonucleotides within the oligonucleotide pool.

In one embodiment, in order to convert a catalytic nucleic acid sensor molecule into an optical nucleic acid sensor molecule, at least a portion of the catalytic domain is

modified (e.g., deleted). In one embodiment, the deletion enhances the conformational

stability of the optical nucleic acid sensor molecule in either the bound or unbound forms.

In one embodiment, shown in Figures 6A and B, deletion of the entire catalytic domain of

the catalytic NASM shown in Figure 5 is shown to stabilize the unbound form of the nucleic

5   acid sensor molecule. In another embodiment, the deletion may be chosen so as to take

advantage of the inherent fluorescence-quenching properties of unpaired guanosine (G)

residues (Walter, N.G. and Burke, J.M., "Real-time monitoring of hairpin ribozyme kinetics

through base-specific quenching of fluorescein-labeled substrates", RNA 3:392 (1997).)

In another embodiment, the target modulation domain from a previously identified

10   nucleic acid sensor molecule is incorporated into an oligonucleotide sequence that changes

conformation (e.g., from a duplexed hairpin to a G-quadruplex) upon target binding. Optical

Nucleic acid sensor molecules of this type can be derived from allosteric ribozymes, such as

those derived from the hammerhead, hairpin, L1 ligase, or group 1 intron ribozymes and the

like, all of which transduce molecular recognition into a detectable signal. For example,

15   3',5'-cyclic nucleotide monophosphate (cNMP)-dependent hammerhead ribozymes were

reengineered into (RNA) optical nucleic acid sensor molecules which specifically bound to

cNMP (Garretta et al., 2001). The catalytic cores for hammerhead ribozymes were removed

and replaced with 5-base duplex forming sequences. The binding of these reengineered

RNA optical NASMs to c-NMP was then confirmed experimentally. By adjusting the

20   duplex length, they can be redesigned to undergo significant conformational changes. The

conformational changes can be coupled to detection via FRET or simply changes in

fluorescence intensity (as in the case of a molecular beacon). For example, by adding an

appropriate probe on the each end of the duplex, the stabilization of duplex by target

binding can be monitored with the change in fluorescence.

25   While the above experimental example is performed in solution and utilizes a

cuvette-based fluorescence spectrometer, in alternative embodiments the methods are

performed in microwell multiplate readers (e.g., the Packard Fusion, or the Tecan Ultra) for

high-throughput solution phase measurements.

In another embodiment, a nucleic acid sensor molecule is bound to a surface by a

30   linker attached to one end of the molecule. For example, a nucleic acid sensor molecule is

modified to include a 12 carbon linker terminated with an amine group. This free amine

group allows the NASM to be attached to an aldehyde-derivatized glass surface via standard

protocols for Schiff base formation and reduction. The nucleic acid sensor molecules can

be bound in discrete regions or spots to form an array or uniformly distributed to cover an

extended area. In the absence of target molecule, the optical nucleic acid sensor molecule forms a stem-loop conformation with duplex formation along the stem due to the complementarity of the nucleotides at the 3'and 5' ends of the molecule. In the presence of the target molecule, the optical nucleic acid sensor molecule undergoes a conformational

5   rearrangement. In some embodiments, this conformational rearrangement results in a change in the distance between the fluorophore attached to the 5' end and the quencher attached to the 3' end. With the quencher separated from the fluorophore, the detected fluorescence emission intensity from the fluorophore increases sharply. The detected increase in fluorescence intensity with target molecule concentration can be used to detect

10   and quantify the amount of target present in a sample solution introduced onto the surface. A sample solution can be laterally confined about the sensor surface by a coverslip, microwell, incubation chamber seal, or flowcell.

In one embodiment, after deletion of at least a portion of the catalytic site from a catalytic nucleic acid sensor molecule, an optical signaling unit is either added to, or

15   inserted within, the nucleic sensor molecule, generating a sensor molecule whose optical properties change in response to binding of the target molecule to the target modulation domain. In one embodiment, the optical signaling unit is added by exposing at least a 5' or 3' nucleotide that was not previously exposed. The 5' nucleotide or a 5' subterminal nucleotide (e.g., an internal nucleotide) of the molecule is couplable to a first signaling

20   moiety while the 3' nucleotide or 3' subterminal nucleotide is couplable to a second signaling moiety. Target molecule binding to the optical nucleic acid sensor molecule alters the proximity of the 5' and 3' nucleotide (or subterminal nucleotides) with respect to each other, and when the first and second signaling moieties are coupled to their respective nucleotides, this change in proximity results in a target sensitive change in the optical

25   properties of the nucleic acid sensor molecule. Detection of changes in the optical properties of the nucleic acid sensor molecule can therefore be correlated with the presence and/or quantity of a target molecule in a sample.

In another embodiment optical NASMs are generated by adding first and second signaling moieties, that are coupled to the 5' terminal or subterminal sequences, and 3 '-

30   terminal and subterminal sequences respectively, of the catalytic NASM. Signaling molecules can be coupled to nucleotides which are already part of the nucleic acid sensor molecule or may be coupled to nucleotides which are inserted into the nucleic acid sensor molecule, or can be added to a nucleic acid sensor molecule as it is synthesized. Coupling chemistries to attach signaling molecules are well known in the art (see, for example, The

Molecular Probes Handbook, R. Haughland). Suitable chemistries include, *e.g.*, derivatization of the 5-position of pyrimidine bases (*e.g.*, using 5'-amino allyl precursors), derivatization of the 5'-end (*e.g.*, phosphoroamidites that add a primary amine to the 5'-end of chemically-synthesized oligonucleotide) or the 3'-end (*e.g.*, periodate treatment of RNA
5   to convert the 3'-ribose into a dialdehyde which can subsequently react with hydrazide-bearing signaling molecules).

     In another embodiment, a single signaling moiety is either added to, or inserted within, the catalytic nucleic sensor molecule. In this embodiment, binding of the target molecule results in changes in both the conformation and physical aspect (*e.g.*, molecular
10   volume, and thus rotational diffusion rate, etc.) of the optical nucleic acid sensor molecule. Conformational changes in the optical nucleic acid sensor molecule upon target binding will modify the chemical environment of the signaling moiety, while changes in the physical aspect of the nucleic acid sensor molecule will alter the kinetic properties of the signaling moiety. In both cases, the result will be a detectable change in the optical properties of the
15   nucleic acid sensor molecule.

     In one embodiment, the optical nucleic acid sensor molecule is prepared without a quencher group. Instead of a quencher group a moiety with a free amine group can be added. This free amine group allows the sensor molecule to be attached to an aldehyde-derivatized glass surface via standard protocols for Schiff base formation and reduction. The
20   nucleic acid sensor molecules can be bound in discrete regions or spots to form an array, or uniformly distributed to cover an extended area. In the absence of target, the optical nucleic acid sensor molecule will diffusionally rotate about its point of attachment to the surface at a rate characteristic of its molecular volume and mass. After target binding, the optical NASM-target complex will have a correspondingly larger volume and mass. This change in
25   molecular volume (mass) will slow the rate of rotational diffusion, and result in a measurable change in the polarization state of the fluorescence emission from the fluorophore.

     In one embodiment of the invention, a single signaling moiety is attached to a portion of a catalytic NASM that is released as a result of catalysis (*e.g.*, either end of a self-
30   cleaving ribozyme or the pyrophosphate at the 5'-end of a ligase). Target molecule-activated catalysis leads to release of the signaling moiety from the optical NASM to generate a signal correlated with the presence of the target. Release can be detected by either (1) changes in the intrinsic optical properties of the signaling moiety (*e.g.*, decreased fluorescence polarization as the released moiety is able to tumble more freely in solution),

or (2) changes in the partitioning of the signaling moiety (*e.g.*, release of a fluorophore from a chip containing immobilized ribozymes such that the total fluorescence of the chip is reduced following washing).

In another embodiment of the invention, the catalytic nucleic acid sensor molecule is unmodified and the optical signaling unit is provided as a substrate for the NASM. One example of this embodiment includes a fluorescently tagged oligonucleotide substrate which can be joined to a NASM with ligase activity. In a heterogeneous assay using the ligase as a sensor molecule, analyte-containing samples are incubated with the fluorescent oligonucleotide substrate and the ligase under conditions that allow the ligase to function. Following an incubation period, the ligase is separated from free oligonucleotide substrate (*e.g.*, by capturing ligases onto a solid support on the basis of hybridization to ligase-specific sequences or by pre-immobilizing the ligases on a solid support and washing extensively).

Quantitation of the captured fluorescence signal provides a means for inferring the concentration of analyte in the sample. In a second example of this embodiment, catalytic activity alters the fluorescence properties of a oligonucleotide substrate without leading to its own modification. Fluorophore pairs or fluorophore/quencher pairs can be attached to nucleotides flanking either side of the cleavage site of an oligonucleotide substrate for a trans-acting endonuclease ribozyme (Jenne et al.). Target activated cleavage of the substrate leads to separation of the pair and a change in its optical properties.

In another embodiment of the invention, the ligase catalytic NASM and its oligonucleotide substrates are unmodified and detection relies on catalytically-coupled changes in the ability of the NASM to be enzymatically amplified. In one example, a target-activated ligase is incubated together with oligonucleotide substrate and an analyte-containing sample under conditions which allow the ligase to function. Following an incubation period, the reaction is quenched and the mixture subjected to RT/PCR amplification using a primer pair that includes the oligo sequence corresponding to the ligation substrate. Amplification products can be detected by a variety of generally practiced methods (*e.g.* Taqman®). Only those ribozymes that have self-ligated an oligonucleotide substrate are capable of amplification under these conditions and will generate a signal that can be coupled to the concentration of the sensor target.

i.   **Proximity Dependent Signaling Moieties**

Many proximity dependent signaling moieties are known in the art and are encompassed within the scope of the present invention (*see, e.g.*, Morrison, 1992, in Nonisotopic DNA Probe Techniques, Kricka, ed., Academic Press, Inc., San Diego, Calif.,

5   chapter 13; and Heller and Morrison, 1985, in Rapid Detection and Identification of Infectious Agents, Academic Press, Inc., San Diego, Calif., pages 245-256). Systems using these signaling moieties rely on the change in fluorescence that occurs when the moieties are brought into close proximity. Such systems are described in the literature as fluorescence energy transfer (FET), fluorescence resonance energy transfer (FRET),

10   nonradiative energy transfer, long-range energy transfer, dipole-coupled energy transfer, or Forster energy transfer (*see, e.g.*, U.S. Patent Number 5,491,063, Wu, and Brand, 1994).

Other proximity-dependent signaling systems that do not rely on direct energy transfer between signaling moieties are also known in the art and can be used in the methods described herein. These include, *e.g.*, systems in which a signaling moiety is

15   stimulated to fluoresce or luminesce upon activation by the target molecule. This activation may be direct (*e.g.*, as in the case of scintillation proximity assays (SPA), via a photon or radionuclide decay product emitted by the bound target), or indirect (*e.g.*, as in the case of AlphaScreen™ assays, via reaction with singlet oxygen released from a photosensitized donor bead upon illumination). In both scenarios, the activation of detected signaling

20   moiety is dependent on close proximity of the signaling moiety and the activating species. In general, for both fluorescence, fluorescence polarization, and scintillation-proximity-type assays, the nucleic acid sensor molecule may be utilized in either solution-phase or solid-phase formats. That is, in functional form, the nucleic acid sensor molecule may be tethered (directly, or via a linker) to a solid support or free in solution.

25   In one embodiment, a scintillation proximity assay (SPA) is used. In this embodiment, the nucleic acid sensor molecules, ligate on oligonucleotide substrate in the presence of a target molecule (*see* Figures 2A and B), are bound to a scintillant-impregnated microwell plate (*e.g.*, FlashPlates, NEN Life Sciences Products , Boston, MA) coated with, for example, streptavidin via a (biotin) linker attached to the 5' end of the effector

30   oligonucleotide sequence (for example, GCGACTGGACATCACGAG (SEQ ID NO:51) in Figure 2A). The various plate-sensor coupling chemistries are determined by the type and manufacturer of the plates, and are well-known in the art. Upon the addition of a solution containing target molecule and excess radiolabeled (*e.g.*, with ^{32}P or ^{35}S) oligonucleotide

substrate in ligation buffer, the NASMs hybridize and ligate the substrate oligonucleotide. Some fraction of the radiolabeled oligonucleotide substrate will be ligated to surface-immobilized NASMs on the plate, while unligated oligonucleotide substrate will be free in solution. Only those oligonucleotide substrates ligated to surface-immobilized NASMs on

5   the plate will be in close enough proximity to the scintillant molecules embedded in the plate to excite them, thereby stimulating luminescence which can be easily detected using a luminometer (e.g., the TopCount luminescence plate reader, Packard Biosciences, Meriden, CT). This type of homogeneous assay format provides straightforward, real-time detection, quantification, and kinetic properties of target molecule binding.

10   In another embodiment, a similar SPA assay format is performed using scintillant-impregnated beads (e.g., Amersham Pharmacia Biotech, Inc., Piscataway, NJ). In this embodiment, the NASMs which ligate on oligonucleotide substrate in the presence of a target molecule (see Figures 2A and B) are coupled to scintillant-impregnated beads which are suspended in solution in, for example, a microwell plate. The various bead-sensor

15   coupling chemistries are determined by the type and manufacturer of the beads, and are well-known in the art. Upon the addition of a solution containing target molecule and excess radiolabeled (e.g., with ^{32}P or ^{35}S) oligonucleotide substrate in ligation buffer, the NASMs hybridize and ligate the oligonucleotide substrate. Some fraction of the radiolabeled substrate will be ligated to surface-immobilized NASMs on the beads, while unligated

20   substrate will be free in solution. Only those substrates ligated to surface-immobilized NASMs on the beads will be in close enough proximity to the scintillant molecules embedded in the beads to excite them, thereby stimulating luminescence which can be easily detected using a luminometer (e.g., the TopCount luminescence plate reader, Packard Biosciences, Meriden, CT). In addition to enabling real-time target detection and

25   quantification, this type of homogeneous assay format can be used to investigate cellular processes in situ in real time. This could be done by culturing cells directly onto a microwell plate and allowing uptake of scintillant beads and radioisotope by cells. Biosynthesis, proliferation, drug uptake, cell motility, etc. can then be monitored via the luminescence signal generated by beads in presence of selected target molecules (see Cook et al.,1992, or

30   Heath et al., 1992).

          Figures 11 A and B show an exemplary embodiment of a non-isotopic proximity assay based on nucleic acid sensor molecules used in conjunction with AlphaScreen™ beads (Packard Biosciences, Meriden, CT). In this embodiment, the nucleic acid sensor

molecules, which ligate on oligonucleotide substrate in the presence of a target molecule (see Figures 2A and B), are bound to a chemiluminescent compound-impregnated acceptor bead coated with, for example, streptavidin, via a (biotin) linker attached to the 5' end of the effector oligonucleotide sequence (GCGACTGGACATCACGAG (SEQ ID NO:51) in

5 Figure 2A). The various bead-sensor coupling chemistries are determined by the type and manufacturer of the beads, and are well-known in the art. The oligonucleotide substrate is coupled to a photosensitizer-impregnated donor bead coated with, for example, streptavidin, via a (biotin) linker attached to the 3' end of the substrate. The donor (substrate) and acceptor (ribozyme) beads and target molecules are then combined in solution in a

10 microwell plate, some of the NASMs hybridize and ligate the oligonucleotide substrate, bringing the donor and acceptor beads into close proximity (< 200 nm). Upon illumination at 680 nm, the photosensitizer in the donor bead converts ambient oxygen into the singlet state at a rate of approximately 60,000/second per bead. The singlet oxygen will diffuse a maximum distance of approximately 200 nm in solution; if an acceptor bead containing a

15 chemiluminescent compound is within this range, i.e., if ligation has occurred in the presence of the target molecule, chemiluminescence at 370 nm is generated. This radiation is immediately converted within the acceptor bead to visible luminescence at 520-620 nm with a decay half-life of 0.3 sec. The visible luminescence at 520-620 nm is detected using a time-resolved fluorescence/ luminescence plate reader (e.g., the Fusion multifunction plate

20 reader, Packard Biosciences, Meriden, CT). This type of nonisotopic homogeneous proximity assay format provides highly sensitive detection and quantification of target molecule concentrations in volumes < 25 microliters for high throughput screening (see Beaudet et al. 2001).

Suitable fluorescent labels are known in the art and commercially available from, for

25 example, Molecular Probes (Eugene, Oreg.). These include, e.g., donor/acceptor (i.e., first and second signaling moieties) molecules such as: fluorescein isothiocyanate (FITC) /tetramethylrhodamine isothiocyanate (TRITC), FITC/Texas Red TM Molecular Probes), FITC/N-hydroxysuccinimidyl 1-pyrenebutyrate (PYB), FITC/eosin isothiocyanate (EITC), N-hydroxysuccinimidyl 1-pyrenesulfonate (PYS)/FITC, FITC/Rhodamine X (ROX),

30 FITC/tetramethylrhodamine (TAMRA), and others. In addition to the organic fluorophores already mentioned, various types of nonorganic fluorescent labels are known in the art and are commercially available from, for example, Quantum Dot Corporation, Inc. Hayward CA). These include, e.g., donor/ acceptor (i.e., first and second signaling moieties) semiconductor nanocrystals (i.e., 'quantum dots') whose absorption and emission spectra

can be precisely controlled through the selection of nanoparticle material, size, and composition (see, for example, Bruchez et al., 1998, Chan and Nie, 1998, Han et al., 2001).

The selection of a particular donor/acceptor pair is not critical to practicing the invention provided that energy can be transferred between the donor and the acceptor. P-(dimethyl aminophenylazo) benzoic acid (DABCYL) is one example of a non-fluorescent acceptor dye which effectively quenches fluorescence from an adjacent fluorophore, e.g., fluorescein or 5-(2'-aminoethyl) aminonaphthalene (EDANS).

Figures 3A and B and 6A and B show exemplary optical nucleic acid sensor molecules derived from catalytic nucleic acid molecules (Figures 2A and B and 5, respectively), according to two embodiments. Figure 3 shows a catalytic nucleic acid sensor molecule obtained from an oligonucleotide pool in which the catalytic site was a ligase site. Figure 6 shows a catalytic nucleic acid sensor molecule obtained from an oligonucleotide pool in which the catalytic site was a site mediating self-cleavage.

In the embodiment shown in Figures 3A and B, a catalytic nucleic acid sensor molecule from which a portion of a ligase site (e.g., the AGUCG sequence at the 3' end of the nucleic acid sensor precursor molecule, as shown in Figure 2) has been removed is coupled to a first signaling moiety (F) at a first nucleotide (1) and to a second signaling moiety (D) at a second nucleotide (2). In a further embodiment, the first and second signaling moieties molecules are attached to non-terminal sequences. The position of the non-terminal sequences coupled to signaling moieties is limited to a maximal distance from the 5' or 3' nucleotide which still permits proximity dependent changes in the optical properties of the molecule. Coupling chemistries are routinely practiced in the art, and oligonucleotide synthesis services provided commercially (e.g., Integrated DNA Technologies, Coralville, IA) can also be used to generate labeled molecules. In a further embodiment, the nucleic acid sensor molecule is used, either tethered to a solid support or free in solution, to detect the presence and concentration of target molecules in a complex biological fluid.

In the embodiment shown in Figures 3A and B, the first signaling moiety (F) is a fluorescein molecule coupled to the 5' end and the second signaling molecule (D) is a DABCYL molecule (a quenching group) coupled to the 3' end. Because of the nearly complete base pairing of the non-target molecule activated form (see Figure 3B), this is the favored form of the nucleic acid sensor molecule in the absence of the target molecule. When the nucleic acid sensor molecule is not activated by target molecule, the fluorescent group and the quenching group are in close proximity and little fluorescence is detectable

from the fluorescent group. Addition of target molecule causes a change in the conformation of the optical nucleic acid sensor molecule shown in Figure 3B to that shown in Figure 3A. When the molecule assumes the conformation shown in Figure 3A, the first and second signaling moieties (F and D, respectively) are no longer in sufficient proximity

5   for the quenching group to quench the fluorescence of the fluorescent group, resulting in a detectable fluorescent signal being produced upon recognition of the target molecule.

In one embodiment, the target modulation domain sequence from a previously identified nucleic acid sensor molecule is incorporated into a separate oligonucleotide sequence which changes conformation upon target recognition as shown in Figures 6A and

10   B. During or after synthesis, an optical signal generating unit is either added or inserted into the oligonucleotide sequence comprising the derived nucleic acid sensor molecule. As noted previously, nucleic acid sensor molecules of this type can be derived from allosteric ribozymes, such as those derived from the hammerhead, hairpin, L1 ligase, or group 1 intron ribozymes and the like (see Soukup et al., 2001, or Hamaguchi et al., 2001), all of

15   which transduce molecular recognition into a detectable optical signal.

In the embodiment shown in Figures 9 A, B, and C, a self-cleaving ribozyme such as the hammerhead (in this case attached to a solid support via a linker molecule is shown) is labeled with a fluorphore. In Figure 9 A, the labeled NASM in the unactivated state comprises two oligonucleotide including a transacting cleavage substrate which bears a

20   second fluorescent label. In the unactivated state, *i.e.*, in the absence of target molecule, the donor fluorophore (on one oligonucleotide NASM) and the acceptor fluorophore (on the cleavage substrate) are in sufficiently close proximity for FRET to occur; thus, minimal fluorescent emission is detected from the donor fluorophore at wavelength 3, $\lambda 3$, upon epi-illumination excitation at wavelength 1, $\lambda 1$. Upon target molecule recognition, the cleavage

25   fragment of the cleavage substrate bearing the acceptor fluorophore dissociates from the ribozyme-target complex. Once separated from the acceptor fluorophore, the donor fluorophore can no longer undergo de-excitation via FRET, resulting in a detectable increase in its fluorescent emission at wavelength 2, $\lambda 2$ (see, for example, Singh. et al., 1999; Wu, and Brand, 1994; Walter and Burke, 1997; Walter et al., 1998). In a further

30   embodiment, the change in the polarization state of the fluorescent emission from the donor fluorophore (due to the increased diffusional rotation rate of the smaller cleavage fragment) can be detected/monitored in addition to changes in fluorescent emission intensity (see, for Singh, 2000). In a further embodiment, the NASMs are free in solution.

In another embodiment, shown in Figure 9 B, the acceptor fluorophore attached to the cleavage substrate is replaced by a quencher group. This replacement will also result in minimal fluorescent donor emission at wavelength 2, $\lambda 2$, when the NASM is in the unbound state under epi-illumination excitation at wavelength 1, $\lambda 1$. Upon target molecule

5   recognition, the cleavage fragments of the cleavage substrate bearing the donor and quencher groups dissociate from the NASM-target molecule complex. Once separated from the quencher, the donor fluorophore will exhibit a detectable increase in its fluorescent emission at wavelength 2, $\lambda 2$. In a further embodiment, the change in the polarization state of the fluorescent emission from the donor fluorophore (due to the increased diffusional

10   rotation rate of the smaller cleavage fragment) can be detected/monitored in addition to changes in fluorescent emission intensity. In a further embodiment, NASMs are free in solution.

In a different embodiment, the optical configuration is designed to provide excitation via total internal reflection (TIR)-illumination, as shown in Figure 9 C. Also, the

15   donor fluorophore is attached to the NASM body while the quencher is attached to the cleavage substrate. In this configuration, with the surface-immobilized NASM in the unbound state, the fluorescent donor emission at wavelength 2, $\lambda 2$, will be minimal. Upon target module recognition, the cleavage fragment of the cleavage substrate bearing the quencher group dissociates from the NASM-target module complex. Once separated from

20   the quencher, the donor fluorophore will exhibit a detectable increase in its fluorescent emission at wavelength 2, $\lambda 2$. In an alternative embodiment to that shown in shown in Figure 9 C, the quencher group can be replaced with an acceptor fluorophore. In yet another alternative embodiment to those shown in Figures 9 A, B, and C, the donor fluorophore is coupled to the cleavage fragment of the cleavage substrate and the acceptor fluorophore or

25   quencher group is deleted. Upon target molecule recognition and dissociation of the cleavage fragment, the polarization state of the fluorescent emission from the donor fluorophore will undergo a detectable change due to the difference in the diffusional rotation rates of the surface-bound ribozyme-target complex and the free cleavage fragment.

In one embodiment, a universal FRET trans-substrate is synthesized for all NASMs

30   derived from self-cleaving allosteric ribozymes. This substrate would have complementary optical signaling units (*i.e.*, donor and acceptor groups) coupled to opposite ends of the synthetic oligonucleotide sequence. Such a universal substrate would obviate the need for coupling optical signaling units to the sensor (*i.e.*, ribozyme) molecule itself.

The relative stabilities of the activated and unactivated forms of the nucleic acid
sensor molecules is optimized to achieve the highest sensitivity of detection of target
molecule. In one embodiment, the nucleic acid sensor molecule is further engineered to
enhance the stability of one form over another. In one embodiment, the boxed UA in

5   Figures 3A and B is changed to a CC, favoring the formation of the target molecule
activated form. Because these bases do not form base pairs when the nucleic acid sensor
molecule is unactiaved, the unactivated form is not stabilized.

In addition to the herein described methods, any additional proximity dependent
signaling system known in the art can be used to practice the method according to the

10   invention, and are encompassed within the scope .

A number of methods can be used to evaluate the relative stability of different
conformations of the nucleic acid sensor molecule. In one embodiment, the free energy of
the structures formed by the nucleic acid sensor molecule is determined using software
programs such as mfold®, which can be found on the Rensselaer Polytechnic Institute (RPI)

15   web site (www.rpi.edu/dept.).

In another embodiment, a gel assay is performed which permits detection of
different conformations of the nucleic acid sensor molecule. In this embodiment, the nucleic
acid sensor molecule is allowed to come to equilibrium at room temperature or the
temperature at which the nucleic acid sensor molecule will be used. The molecule is then

20   cooled to 4 °C and electrophoresed on a native (non-denaturing) gel at 4 °C. Each of the
conformations formed by the nucleic acid sensor molecule will run at a different position on
the gel, allowing visualization of the relative concentration of each conformation. Similarly,
the conformation of nucleic acid sensor molecules which form in the presence of target
molecule is then determined by a method such as circular dichroism (CD). By comparing

25   the conformation of the nucleic acid sensor molecule formed in the presence of target
molecule with the conformations formed in the absence of target molecule, the
conformation which corresponds to the activated conformation can be identified in a sample
in which there is no target molecule. The nucleic acid sensor molecule can then be
engineered to minimize the formation of the activated conformation in the absence of target

30   molecule. The sensitivity and specificity of nucleic acid sensor molecule can be further
tested using target molecule modulation assays with known amounts of target molecules.

In another embodiment, shown in Figures 6 A and B, a catalytical nucleic acid
sensor molecule from which a portion of a self-cleaving site has been removed, is coupled
to a first signaling moiety (F) at a first nucleotide and to a second signaling moiety (D) at a

second nucleotide. In this embodiment, the entire catalytic site of the catalytic nucleic acid molecule (see Figure 5) has been removed. In one embodiment (Figures 6 A and B), additional bases (*e.g.*, UGGUAU) are added to one end of the portion of the nucleic acid sensor molecule comprising the modulation domain, to stabilize the unbound form of the

5   nucleic acid sensor molecule (Figure 6B). These bases are selected to be complementary to bases at the opposite end of the nucleic acid sensor molecule (ACCAUA). Additional bases may be added to either the 5' or the 3' end of the nucleic acid sensor molecule.

Modifications to stabilize one conformation of the nucleic sensor molecule over another may be identified using the mfold program or native gel assays discussed above. A

10   labeled nucleic acid sensor molecule is generated by coupling a first signaling moiety (F) to a first nucleotide and a second signaling moiety (D) to a second nucleotide as discussed above. As above, the sensitivity and specificity of the nucleic acid sensor molecule can be further assayed by using target molecule modulation assays with known amounts of target molecules.

15   ii.   Optical Signal Generating Units With Single Signaling Moieties

In one embodiment, the optical nucleic acid sensor molecule comprises an optical signaling unit with a single signaling moiety introduced at either an internal or terminal position within the nucleic acid sensor molecule. In this embodiment, binding of the target molecule results in changes in both the conformation and physical aspect (*e.g.*, molecular

20   volume or mass, rotational diffusion rate, etc.) of the nucleic acid sensor molecule. Conformational changes in the nucleic acid sensor molecule upon target recognition will modify the chemical environment of the signaling moiety. Such a change in chemical environment will in general change the optical properties of the signaling moiety. Suitable signaling moieties are described in Jhaveri, et al, 2000, and include, *e.g.*, fluorescein,

25   acridine, and other organic and nonorganic fluorophores.

In one embodiment, a signaling moiety is introduced at a position in the catalytic nucleic acid molecule near the target activation site (identifiable by footprinting studies, for example). Binding of the target molecule will (via a change in conformation of the nucleic acid molecule) alter the chemical environment and thus affect the optical properties of the

30   signaling moiety in a detectable manner.

Recognition of the target molecule by the NASM will result in changes in the conformation and physical aspect of the nucleic acid sensor molecule, and will thus alter the kinetic properties of the signaling moiety. In particular, the changes in conformation and

mass of the sensor-target complex will reduce the rotational diffusion rate for the sensor-target complex, resulting in a detectable change in the observed steady state fluorescence polarization (FP) from the signaling moiety. The expected change in FP signal with target , concentration can be derived using a modified form of the well-known Michaelis-Menten

5   model for ligand binding kinetics (Lakowicz, 1999). FP is therefore a highly sensitive means of detecting and quantitatively determining the concentration of target molecules in a sample solution (Jameson and Sawyer, 1995; Jameson and Seifried, 1999; Jolley, 1999; Singh, 2000; Owicki et al., 1997). FP methods are capable of functioning in both solution- and solid-phase implementations.

10   Numerous additional methods can be used that, *e.g.*, make use of a single fluorescent label and an unpaired guanosine residue (instead of a quencher group), to enable the use of FRET in target detection and quantitation as described in the embodiments above (*see* Walter and Burke, 1997).

        In a further embodiment, shown in Figure 10 A, B, and C an unlabeled ligating

15   ribozyme such as the lysozyme-dependent L1 ligase is shown (*see*, for example, Robertson, M.P. and Ellington, A.D, 2000). In the unactivated state, *i.e.*, in the absence of target, no fluorescent emission is detected from the surface-bound ribozymes under total internal reflection (TIR)-illumination (see Figure 10 A), or epi-illumination (see Figure 10 B). Upon recognition of target molecules in the presence of an oligonucleotide substrate with a tag

20   (where the tag is capable of binding to a subsequently added fluorescent label via interactions including, but not limited to, biotin/streptavidin, amine/aldehyde, hydrazide, thiol, or other reactive groups) those oligonucleotide substrates hybridized to NASMs will undergo ligation and become covalently bonded to the thereto. In order to maximize the probability of hybridization for a given NASM, oligonucleotide substrate can be added in

25   excess relative to NASM, the temperature of the ambient solution in which the reaction takes place can be kept below room temperature (*e.g.*, 4 °C), and agitation of the reaction vessel can be employed to overcome the kinetic limitation of diffusion-limited transport of species in solution. Given the above conditions, as well as sufficient time for maximal hybridization and subsequent ligation to occur, fluorescent label with the appropriate

30   reactive group to bind the substrate tag is added to the reaction mixture. Again, the degree of substrate-label binding can be maximized through control of label concentration, solution temperature, and agitation. Once the fluorescent label has bound to all available ligated substrate-ribozyme-target complex, the solution temperature can be raised to drive off all of the hybridized but unligated substrate. With TIR-illumination, the spatial extent of the

excitation region above the solid substrate surface to which the ribozymes are bound is only on the order of 100 nm. Therefore, the bulk solution above the substrate surface is not illuminated and the detected fluorescent emission will be primarily due to fluorophores which are bound to ligated oligonucleotide substrate-NASM- target molecule complexes

5 tethered to the substrate surface. The fluorescence emission from surface-bound NASM-target molecule complexes in this homogeneous solid phase assay format represents an easily detectable optical signal. In another embodiment, the fluorescence polarization (FP) of the labeled substrate can be monitored, as shown in Figure 10 C. Upon ligation, the steady state fluorescence polarization signal from the substrate-NASM complex will

10 increase detectably relative to the FP signal from the free labeled oligonucleotide substrate in solution, due to the difference in the diffusional rotation rates between the free and ligated forms.

In another embodiment, an unlabeled ligating ribozyme such as the lysozyme-dependent L1 ligase (see, for example, Robertson, M.P. and Ellington, A.D, 2000) is bound

15 to a solid surface. In this embodiment, the oligonucleotide substrate is coupled to an enzyme-linked luminescent moiety, such as horse radish peroxidase (HRP) by a tag (where the tag is capable of binding to a subsequently added label via interactions including, but not limited to, biotin/streptavidin, amine/aldehyde, hydrazide, thiol, or other reactive groups). In the absence of target molecule, no luminescent emission is detected from the

20 surface-bound ribozymes. Upon recognition of target molecules in the presence of labeled oligonucleotide substrate, those oligonucleotide substrates hybridized to NASMs will undergo ligation and become covalently bonded to the NASMs. After removal of excess, unbound oligonucleotide substrate, the activation empty for the enzyme-linked luminescent label is added to the reaction volume. The resulting luminescent signal (e.g., from HRP,

25 luciferase, etc.) is easily detectable using standard luminometers (e.g., the Fusion multifunction plate reader, Packard Bioscience). In a further embodiment, the activated solution can be precipitated, followed by colorimetric detection. In a particular embodiment, the enzyme linked signal amplification, TSA, (sometimes referred to as CARD-catalyzed reporter deposition) is an ultrasensitive detection method. The technology

30 uses turnover of multiple tyramide substrates per horseradish peroxidase (HRP) enzyme to generate high-density labeling of a target protein or nucleic acid probe in situ. Tyramide signal amplification is a combination of three elementary processes: (1) Ligation (or not) of a biotinylated ligase oligonucleotide substrate oligo, followed by binding (or not) of a streptavidin-HRP to the probe; (2) HRP-mediated conversion of multiple copies of a

fluorescent tyramide derivative to a highly reactive radical; (3) Covalent binding of the
reactive, short lived tyramide radicals to nearby nucleophilic residues, greatly reducing
diffusion-related signal loss.

5   2.   Generating Biosensors

Optical nucleic acid sensor molecules for the detection of a target molecule of
interest are generated by first selecting catalytic nucleic acid molecules with catalytic
activity modifiable (e.g., activatable) by a selected target molecule. In one embodiment, at
10   least a portion of the catalytic site of the catalytic NASM is then removed and an optical
signal generating unit is either added or inserted. Recognition of the target molecule by the
nucleic acid sensor molecule activates a change in the properties of the optical signaling
unit.

In one embodiment, a biosensor is provided which comprises a plurality of optical
15   nucleic acid sensor molecules labeled with first and second signaling moieties specific for a
target molecule. In another embodiment, the optical NASMs are labeled with a single
signaling moiety. In one embodiment, the labeled nucleic acid sensor molecules are
provided in a solution (e.g., a buffer). In another embodiment, the labeled nucleic acid
sensor molecules are attached directly or indirectly (e.g., through a linker molecule) to a
20   substrate. In further embodiments, nucleic acid sensor molecules can be synthesized directly
onto the substrate. Suitable substrates which are encompassed within the scope include,
e.g., glass or quartz, silicon, encapsulated or unencapsulated semiconductor nanocrystal
materials (e.g., CdSe), nitrocellulose, nylon, plastic, and other polymers. Substrates may
assume a variety of configurations (e.g., planar, slide shaped, wafers, chips, tubular, disc-
25   like, beads, containers, or plates, such as microtiter plates, and other shapes).

Numerous attachment chemistries, both direct and indirect, can be used to immobilize
the sensor molecules on a solid support. These include, e.g., amine/aldehyde,
biotin/streptavidin (avidin, neutravidin), ADH/oxidized 3' RNA. In a particular
embodiment, the nucleic acid sensor molecules ligate a substrate in the presence of a target
30   molecule (see Figures 2A and B). In this embodiment the ribozymes are bound to a solid
substrate via the effector oligonucleotide sequence (for example,
GCGACTGGACATCACGAG (SEQ ID NO:51) in Figure 2A).

In one embodiment, a manual or computer-controlled robotic microarrayer is used to
generate arrays of nucleic acid sensor molecules immobilized on a solid substrate. In one

embodiment, the arrayer utilizes contact-printing technology (*i.e.*, it utilizes printing pins of metal, glass, etc., with or without quill-slots or other modifications). In a different embodiment, the arrayer utilizes non-contact printing technology (*i.e.*, it utilizes ink jet or capillary-based technologies, or other means of dispensing a solution containing the

5   material to be arrayed). Numerous methods for preparing, processing, and analyzing microarrays are known in the art (*see* Schena et al., 2000; Mace et al., 2000; Heller et al., 1999; Basararsky et al., 2000; Schermer, 1999). Robotic and manual arrayers are commercially available for example, the SpotArray from Packard Biosciences, Meriden, CT, and the RA-1 from GenomicSolutions, Ann Arbor, MI).

10   In one embodiment, larger substrates can be generated by combining a plurality of smaller biosensors forming an array of biosensors. In a further embodiment, nucleic acid sensor molecules placed on the substrate are addressed (*e.g.*, by specific linker or effector oligonucleotide sequences on the nucleic acid sensor molecule) and information relating to the location of each nucleic acid sensor molecule and its target molecule specificity is stored

15   within a processor. This technique is known as spatial addressing or spatial multiplexing. Techniques for addressing nucleic acids on substrates are known in the art and are described in, for example, U.S. Patent Number 6,060,252, U.S. Patent Number 6,051,380, U.S. Patent Number 5,763,263, U.S. Patent Number 5,763,175, and U.S. Patent Number 5,741,462.

In one embodiment different nucleic acid sensor molecules are immobilized on a

20   streptavidin-derivatized glass substrate via biotin linkers. The individual sensor spots can be manually arrayed. Solution measurements of target molecule concentration can be made by bathing the surface of the biosensor array in a solution containing the targets (analytes) of interest. In practice this is accomplished either by incorporating the array within a microflowcell (with a flow rate of ~ 25 microliters/min), or by placing a small volume (~ 6-

25   10 microliters) of the target solution on the array surface and covering it with a cover slip. Detection and quantification of target concentration is accomplished by monitoring changes in the fluorescence polarization (FP) signal emitted from the fluorescein label under illumination by 488 nm laser radiation. The rotational diffusion rate is inversely proportional to the molecular volume; thus the rotational correlation time for the roughly

30   20-nucleotide unbound sensor (*i.e.*, in the absence of target molecule) will be significantly less than that for the target-NASM complex. The fluorescence emission from the target-NASM complex will therefore experience greater residual polarization due to the smaller angle through which the emission dipole axis of the sensor fluorophore can rotate within its radiative lifetime. In another embodiment, different surface attachment chemistries are used

to immobilize the NASMs on a solid substrate. As previously noted, these include, *e.g.*, interactions involving biotin/streptavidin, amine/aldehyde, hydrazide, thiol, or other reactive groups.

5   The specificity of the biosensors and NASMs according to the invention is determined by the specificity of the target modulation domain of the nucleic acid sensor molecule. In one embodiment, a biosensor is provided in which all of the nucleic acid sensor molecules recognize the same molecule. In another embodiment, a biosensor is provided which can recognize at least two different target molecules allowing for multi-analyte detection. Multiple analytes can be distinguished by using different combinations of first and second

10   signaling molecules. In addition to the wavelength/color and spatial multiplexing techniques previously described, biosensors may be used to detect multiple analytes using intensity multiplexing. This is accomplished by varying the number of fluorescent label molecules on each biosensor in a controlled fashion. Since a single fluorescent label is the smallest integral labeling unit possible, the number of fluorophores (*i.e.*, the intensity from) a given

15   biosensor molecule provides a multiplexing index. Using the combination of 6-wavelength (color) and 10-level intensity multiplexing, implemented in the context of semiconductor nanocrystals derivatized as bioconjugates, would theoretically allow the encoding of million different analyte-specific biosensors (Han et al., 2001).

        In one embodiment, multiple single target biosensors can be combined to form a

20   multianalyte detection system which is either solution-based or substrate-based according to the needs of the user. In this embodiment, individual biosensors can be later removed from the system, if the user desires to return to a single analyte detection system (*e.g.*, using target molecules bound to supports, or, for example, manually removing a selected biosensor(s) in the case of substrate-based biosensors). In a further embodiment, nucleic

25   acid sensor molecules binding to multiple analytes are distinguished from each other by referring to the address of the nucleic acid sensor molecule on a substrate and correlating its location with the appropriate target molecule to which it binds (previously described as spatial addressing or multiplexing).

        In one embodiment, subsections of a biosensor array can be individually subjected to

30   separate analyte solutions by use of substrate partitions or enclosures that prevent fluid flow between subarrays, and microfluidic pathways and injectors to introduce the different analyte solutions to the appropriate sensor subarray.

        3.   **Nucleic acid sensor molecule and biosensor systems**

In one embodiment, a nucleic acid sensor molecule or biosensor system is provided comprising a nucleic acid sensor molecule in communication with a detector system. In a further embodiment, a processor is provided to process optical signals detected by the detector system. In still a further embodiment, the processor is connectable to a server

5   which is also connectable to other processors. In this embodiment, optical data obtained at a site where the NASM or biosensor system resides can be transmitted through the server and data is obtained, and a report displayed on the display of the off-site processor within seconds of the transmission of the optical data. In one embodiment, data from patients is stored in a database which can be accessed by a user of the system.

10   Data obtainable from the biosensors according to the invention include diagnostic data, data relating to lead compound development, and nucleic acid sensor molecule modeling data (e.g., information correlating the sequence of individual sensor molecules with specificity for a particular target molecule). In one embodiment, these data are stored in a computer database. In a further embodiment, the database includes, along with

15   diagnostic data obtained from a sample by the biosensor, information relating to a particular patient, such as medical history and billing information. Although, in one embodiment, the database is part of the nucleic acid sensor molecule system, the database can be used separately with other detection assay methods and drug development methods.

Detectors used with the nucleic acid sensor molecule systems according to the

20   invention, can vary, and include any suitable detectors for detecting optical changes in nucleic acid molecules. These include, e.g., photomultiplier tubes (PMTs), charge coupled devices (CCDs), intensified CCDs, and avalanche photodiodes (APDs). In one embodiment, a nucleic acid sensor molecule comprising labeled nucleic acid sensor molecules is excited by a light source in communication with the biosensor. In a further embodiment, when the

25   optical signaling unit comprises first and second signal moieties that are donor/acceptor pairs (i.e., signal generation relies on the fluorescence of a donor molecule when it is removed from the proximity of a quencher acceptor molecule), recognition of a target molecule will cause a large increase in fluorescence emission intensity over a low background signal level. The high signal-to-noise ratio permits small signals to be measured

30   using high-gain detectors, such as PMTs or APDs. Using intensified CCDs, and PMTs, single molecule fluorescence measurements have been made by monitoring the fluorescence emission, and changes in fluorescence lifetime, from donor/acceptor FRET pairs (see Sako, et al., 2000; Lakowicz et al, 1991)).

Light sources include, *e.g.*, filtered, wide-spectrum light sources, (*e.g.*, tungsten, or xenon arc), laser light sources, such as gas lasers, solid state crystal lasers, semiconductor diode lasers (including multiple quantum well, distributed feedback, and vertical cavity surface emitting lasers (VCSELs)), dye lasers, metallic vapor lasers, free electron lasers,

5   and lasers using any other substance as a gain medium. Common gas lasers include Argon-ion, Krypton-ion, and mixed gas (*e.g.*, Ar-Kr) ion lasers, emitting at 455, 458, 466, 476, 488, 496, 502, 514, and 528 nm (Ar ion); and 406, 413, 415, 468, 476, 482, 520, 531, 568, 647, and 676 nm (Kr ion). Also included in gas lasers are Helium Neon lasers emitting at 543, 594, 612, and 633 nm. Typical output lines from solid state crystal lasers include 532

10   nm (doubled Nd:YAG) and 408/816 nm (doubled/primary from Ti:Sapphire). Typical output lines from semiconductor diode lasers are 635, 650, 670, and 780 nm.

Excitation wavelengths and emission detection wavelengths will vary depending on the signaling moieties used. In one embodiment, where the first and second signaling moieties are fluorescein and DABCYL, the excitation wavelength is 488 nm and the

15   emission wavelength is 514 nm. In the case of semiconductor nanocrystal-based fluorescent labels, a single excitation wavelength or broadband UV source may be used to excite several probes with widely spectrally separated emission wavelengths (*see* Bruchez et al., 1998; Chan et al., 1998).

In one embodiment, detection of changes in the optical properties of the nucleic acid

20   sensor molecules is performed using any of a cooled CCD camera, a cooled intensified CCD camera, a single-photon-counting detector (*e.g.*, PMT or APD), or other light sensitive sensor. In one embodiment, the detector is optically coupled to the nucleic acid sensor molecule through a lens system, such as in an optical microscope (*e.g.*, a confocal microscope). In another embodiment, a fiber optic coupler is used, where the input to the

25   optical fiber is placed in close proximity to the substrate surface of a biosensor, either above or below the substrate. In yet another embodiment, the optical fiber provides the substrate for the attachment of nucleic acid sensor molecules and the biosensor is an integral part of the optical fiber.

In one embodiment, the interior surface of a glass or plastic capillary tube provides

30   the substrate for the attachment of nucleic acid sensor molecules. The capillary can be either circular or rectangular in cross-section, and of any dimension. The capillary section containing the biosensors can be integrated into a microfluidic liquid-handling system which can inject different wash, buffer, and analyte-containing solutions through the sensor tube. Spatial encoding of the sensors can be accomplished by patterning them longitudinally

along the axis of the tube, as well as radially, around the circumference of the tube interior. Excitation can be accomplished by coupling a laser source (e.g., using a shaped output beam, such as from a VCSEL) into the glass or plastic layer forming the capillary tube. The coupled excitation light will undergo TIR at the interior surface/solution interface of the

5 tube, thus selectively exciting fluorescently labeled biosensors attached to the tube walls, but not the bulk solution. In one embodiment, detection can be accomplished using a lens-coupled or proximity-coupled large area segmented (pixelated) detector, such as a CCD. In a particular embodiment, a scanning (i.e., longitudinal/axial and azimuthal) microscope objective lens/emission filter combination is used to image the biosensor substrate onto a

10 CCD detector. In a different embodiment, a high resolution CCD detector with an emission filter in front of it is placed in extremely close proximity to the capillary to allow direct imaging of the biosensors. In a different embodiment, highly efficient detection is accomplished using a mirrored tubular cavity that is elliptical in cross-section. The sensor tube is placed along one focal axis of the cavity, while a side-window PMT is placed along

15 the other focal axis with an emission filter in front of it. Any light emitted from the biosensor tube in any direction will be collected by the cavity and focused onto the window of the PMT.

In still another embodiment, the optical properties of a nucleic acid sensor molecule are analyzed using a spectrometer (e.g., such as a luminescence spectrometer) which is in

20 communication with the biosensor. The spectrometer can perform wavelength discrimination for excitation and detection using either monochromators (i.e., diffraction gratings), or wavelength bandpass filters. In this embodiment, biosensor molecules are excited at absorption maxima appropriate to the signal labeling moieties being used (e.g., acridine at 450 nm, fluorescein at 495 nm) and fluorescence intensity is measured at

25 emission wavelengths appropriate for the labeling moiety used (e.g., acridine at 495 nm; fluorescein at 515 nm). Achieving sufficient spectral separation (i.e., a large enough Stokes shift) between the excitation wavelength and the emission wavelength is critical to the ultimate limit of detection sensitivity. Given that the intensity of the excitation light is much greater than that of the emitted fluorescence, even a small fraction of the excitation light

30 being detected or amplified by the detection system will obscure a weak biosensor fluorescence emission signal. In one embodiment, the biosensor molecules are in solution and are pipetted (either manually or robotically) into a cuvette or a well in a microtiter plate within the spectrometer. In a further embodiment, the spectrometer is a multifunction plate reader capable of detecting optical changes in fluorescence or luminescence intensity (at

one or more wavelengths), time-resolved fluorescence, fluorescence polarization (FP), absorbance (epi and transmitted), etc., such as the Fusion multifunction plate reader system (Packard Biosciences, Meriden, CT). Such a system can be used to detect optical changes in biosensors either in solution, bound to the surface of microwells in plates, or immobilized

5   on the surface of solid substrate (e.g., a biosensor microarray on a glass substrate). This type of multiplate/multisubstrate detection system, coupled with robotic liquid handling and sample manipulation, is particularly amenable to high-throughput, low-volume assay formats.

In embodiments where nucleic acid sensor molecules are attached to substrates,

10   such as a glass slide or in microarray format, it is desirable to reject any stray or background light in order to permit the detection of very low intensity fluorescence signals. In one embodiment, a small sample volume (~10 nL) is probed to obtain spatial discrimination by using an appropriate optical configuration, such as evanescent excitation or confocal imaging. Furthermore, background light can be minimized by the use of narrow-bandpass

15   wavelength filters between the sample and the detector and by using opaque shielding to remove any ambient light from the measurement system.

In one embodiment, spatial discrimination of nucleic acid sensor molecules attached to a substrate in a direction normal to the interface of the substrate (i.e., excitation of only a small thickness of the solution layer directly above and surrounding the plane of attachment

20   of the biosensor molecules to the substrate surface) is obtained by evanescent wave excitation. This is illustrated in Figure 12. Evanescent wave excitation utilizes electromagnetic energy that propagates into the lower-index of refraction medium when an electromagnetic wave is totally internally reflected at the interface between higher and lower-refractive index materials. In this embodiment a collimated laser beam is incident on

25   the substrate/solution interface (at which the biosensors are immobilized) at an angle greater than the critical angle for total internal reflection (TIR). This can be accomplished by directing light into a suitably shaped prism or an optical fiber. In the case of a prism, as shown in Figure 12, the substrate is optically coupled (via index-matching fluid) to the upper surface of the prism, such that TIR occurs at the substrate/solution interface on which

30   the biosensors are immobilized. Using this method, excitation can be localized to within a few hundred nanometers of the substrate/solution interface, thus eliminating autofluorescence background from the bulk analyte solution, optics, or substrate. Target recognition is detected by a change in the fluorescent emission of the nucleic acid sensor,

whether a change in intensity or polarization. Spatial discrimination in the plane of the interface (*i.e.*, laterally) is achieved by the optical system.

In one embodiment, a large area of the biosensor substrate is uniformly illuminated, either via evanescent wave excitation or epi-illumination from above, and the detected

5   signal is spatially encoded through the use of a pixelated detector, such as CCD camera. An example of this type of uniform illumination/CCD detection system (using epi-illumination)) for the case of microarrayed biosensors on solid substrates is the GeneTAC 2000 scanner (GenomicSolutions, Ann Arbor, MI). In a different embodiment, a small area (*e.g.*, 10 x 10 microns to 100 x 100 microns) of the biosensor substrate is illuminated by a

10   micro-collimated beam or focused spot. In one embodiment, the excitation spot is rastered in a 2-dimensional scan across the static biosensor substrate surface and the signal detected (with an integrating detector, such as a PMT) at each point correlated with the spatial location of that point on the biosensor substrate (*e.g.*, by the mechanical positioning system responsible for scanning the excitation spot). Two examples of this type of moving spot

15   detection system for the case of microarrayed biosensors on solid substrates are: the DNAScope scanner (confocal, epi-illumination, GeneFocus, Waterloo, ON, Canada), and the LS IV scanner (non-confocal, epi-illumination, GenomicSolutions, Ann Arbor, MI). In yet another embodiment, a small area (*e.g.*, 10 x 10 microns to 100 x 100 microns) of the biosensor substrate is illuminated by a stationary micro-collimated beam or focused spot,

20   and the biosensor substrate is rastered in a 2-dimensional scan beneath the static excitation spot, with the signal detected (with an integrating detector, such as a PMT) at each point correlated with the spatial location of that point on the biosensor substrate (*e.g.*, by the mechanical positioning system responsible for scanning the substrate). An example of this type of moving substrate detection (using confocal epi-illumination) system for the case of

25   microarrayed biosensors on solid substrates is the ScanArray 5000 scanner (Packard Biochip, Billerica, MA).

In the embodiment shown in Figure 12, a TIR evanescent wave excitation optical configuration is implemented, with a static substrate and dual-capability detection system. The detection system is built on the frame of a Zeiss universal fluorescence microscope.

30   The system is equipped with 2 PMTs on one optical port, and an intensified CCD camera (Cooke, St. Louis, MO) mounted on the other optical port. The optical path utilizes a moveable mirror which can direct the collimated, polarized laser beam through focusing optics to form a spot, or a beam expander to form a large (> 1cm) beam whose central portion is roughly uniform over the field of view of the objective lens. Another movable

mirror can direct the light either to the intensified CCD camera when using large area uniform illumination, or to the PMTs in the scanned spot mode. In spot scanning mode, a polarizing beamsplitter separates the parallel and perpendicular components of the emitted fluorescence and directs each to its designated PMT. An emission filter in the optical

5   column rejects scattered excitation light from either type of detector. In CCD imaging mode, manually adjusted polarizers in the optical column of the microscope must be adjusted to obtain parallel and perpendicular images from which the fluorescence polarization or anisotropy can be calculated. A software program interfaces with data acquisition boards in a computer which acquires the digital output data from both PMTs and

10   CCD. This program also controls the PMT power, electromechanical shutters, and galvanometer mirror scanner, calculates and plots fluorescence polarization in real time, and displays FP and intensity images.

In another embodiment, the detection system is a single photon counter system (*see*, *e.g.*, U.S. Patent Number 6,016,195 and U.S. Patent Number 5,866,348) requiring rastering

15   of the sensor substrate to image larger areas and survey the different binding regions on the biosensor.

In another embodiment of the invention, the biosensor is used to detect a target molecule through changes in the electrochemical properties of the nucleic acid sensor moleculeor molecules in close proximity to it which occur upon recognition of the NASM

20   to the target molecule.

In a preferred embodiment, the biosensor system would consist of three major components: One, optical nucleic acid sensor molecules immobilized on an array of independently addressable gold electrodes. The nucleic acid sensor molecules immobilized on each electrode may be modulated by the same or different target molecules, including

25   proteins, metabolites and other small molecules, etc.; two, an oligonucleotide substrate which acts as a signaling probe, hybridizing to the oligonucleotide substrate binding domain of the ligase sensor and forming a covalent phosphodiester bond with the nucleic acid sensor molecule nucleotide adjacent to its 3' terminus in the presence of the appropriate target. This oligonucleotide substrate is typically a nucleic acid sequence containing one or

30   more modified nucleotides conjugated to redox active metallic complexes, *e.g.*, ferrocene moieties, which can act as electron donors; and three, an immobilized mixed self-assembled surface monolayer (SAM), comprised of conductive species separated by insulating species, covering the surface of the electrodes. Examples of conductive species include thiol-terminated linear molecules, such as oligophenylethyl molecules, while examples of

nonconductive thiol-terminated linear molecules, include alkane-thiol molecules terminated with polyethylene glycol (PEG). All immobilized species can be covalently attached to the electrode surface by terminal thiol groups. Figure 15 schematically shows the structure of the mixed self-assembled surface monolayer (SAM) coating the gold electrode, as well as

5   the immobilized nucleic acid sensor molecule (NASM) with a ligated oligonucleotide substrate conjugated to several redox active moieties. Upon recognition of the target molecule by the target modulation domain and subsequent ligation of the oligonucleotide substrate at the site indicated in the figure, the redox active signaling moieties coupled to the substrate oligo will be brought into close proximity to the conductive surface layer,

10   resulting in a detectable increase in electronic surface signal.

In another preferred embodiment, the biosensor system would consist of two major components: (1) Optical nucleic acid sensor molecules immobilized on an array of independent addressable gold electrodes. The nucleic acid sensor molecules immobilized on each electrode may be modulated by the same or different target molecules, including

15   proteins, metabolites and other small molecules, etc. The NASM will contain one or more nucleotides conjugated to redox active metallic complexes, e.g., ferrocene moieties, which can act as electron donors; and (2) an immobilized mixed self-assembled surface monolayer (SAM), comprised of conductive species separated by insulating species, covering the surface of the electrodes. Examples of conductive species include thiol-terminated linear

20   molecules, such as oligophenylehtynyl molecules, while examples of nonconductive thiol-terminated linear molecules include alkane-thiol molecules terminated with polyethylene glycol (PEG). Figure 16 shows the SAM-coated molecule immobilized via a capture oligonucleotide. In this case, the redox active signaling moieties are coupled to the body of the NASM, as shown in the figure. Upon recognition of the target molecule by the target

25   modulation domain and subsequent cleavage at the site indicated in the figure, the bulk of the NASM, including the nucleotides coupled to the redox active signaling moieties will dissociate from the surface, resulting in a detectable loss of electronic current signal.

In another embodiment, the array would be subjected, e.g., by an integrated microfluidic flowcell, to an analyte solution containing the target(s) of interest at some

30   unknown concentration. The range of possible sample analyte solutions may include standard buffers, biological fluids, and cell or tissue extracts. The sample solution will also contain the signaling probe at a saturating concentration relative to the immobilized nucleic acid sensor molecule. This ensures that at any given time during analysis, there is a high probability that each nucleic acid sensor molecule will have a signaling probe hybridized to

it. In the presence of the target molecules in the sample solution, the nucleic acid sensor molecule will form a covalent phosphodiester bond, *i.e.*, ligate, with the signaling probe, thus immobilizing it with its redox active electron donor species in electrical contact with the conductive molecules within the mixed self-assembled surface monolayer. After some

5   integration time, during which signal probe ligation occurs, it may be necessary to denature the hybridized but unligated signaling probes. This denaturation step, which effectively removes 'background' signaling probes and their associated redox moieties from the vicinity of the electrode, can be accomplished by a small temperature increase (*e.g.*, from 21 °C to 25 °C), or by a brief negative voltage spike applied to the sensor electrodes followed

10   by the application of a large positive DC voltage to a separate electrode that would collect unligated signaling. For the case of a sufficiently short hybridization region, *e.g.*, 5 base-pairs, on the signaling probe, a separate denaturation step may not be necessary. In either case, following nucleic acid sensor molecule activation by target molecules, a linear electrical potential ramp is applied to the electrodes. The redox species conjugated to the

15   immobilized signaling probe-nucleic acid sensor molecule will be electrochemically oxidized, liberating one or more electrons per moiety. The conductive molecules within the surface monolayer will provide an electrical path for the liberated electrons to the electrode surface, as shown in Figure 17.

The net electron transfer to or from the electrode will be measured as a peak in the

20   faradaic current, centered at the redox potential of the electron donor species (specified for a given reference electrode) and superposed on top of the capacitive current baseline which is observed in the absence of surface-immobilized signaling probes. This is shown schematically in Figure 18.

Quantitative analysis of the sensor signal, and therefore accurate determination of

25   target molecule concentration, is based on the fact that the measured faradaic peak height is directly proportional to number of redox moieties immobilized at the electrode, that is, the number of nucleic acid sensor molecules ligated to signaling probes times the multiplicity of redox moieties per signaling probe molecule. Signal generation by the nucleic acid sensor molecules is thus amplified by virtue of multiple redox species per signaling probe. In

30   addition, if an alternating current (AC) bias voltage is.applied (superposed) on top of the DC linear voltage ramp applied to the sensor electrodes, *i.e.*, in the case of AC voltammetry, signal amplification would result from the cyclic repetition of the signal-generating redox reaction.

The system described above for the case of a surface-immobilized nucleic acid sensor molecule which ligates a signaling probe containing one or more modified nucleotides conjugated to redox active species suggests a general method and instrumentation for the detection and quantitation of an arbitrary target molecule in solution

5   in real time. Detection of a particular target would require development of a nucleic acid sensor molecule against that recognizes the target molecule. Additionally, nucleic acid sensor molecules have been developed which are activated only in the presence of two different target molecules. Such dual-effector sensors could be used to detect the simultaneous presence of two or more targets, or could be used in conjunction with single-

10   target molecule sensors to form biological logic (*i.e.*, AND, OR, etc.) circuits.

Multiplexed detection of multiple target molecules simultaneously in a complex sample solution could be accomplished by immobilizing nucleic acid sensor molecules against the target molecules of interest on separate electrodes within a two-dimensional array of electrodes. A complex sample solution containing multiple target molecules and a

15   common signaling probe could then be introduced to the array. All nucleic acid sensor molecules would be exposed simultaneously to all targets, with the target-activated nucleic acid sensor molecule response(s) being observed and recorded only at the spatial location(s) known to contain a nucleic acid sensor molecule specific for the target molecules present in the (unknown) sample. The utility of such a nucleic acid sensor molecule array would be

20   greatly enhanced by the integration of a microfluidic sample and reagent delivery system. Such an integrated microfluidic system would allow the application of reagents and samples to the sensor array to be automated, and would allow the reduction of sample volume required for analysis to < 1 uL.

The sensor array electrodes may be of any configuration, number, and size. In a

25   preferred embodiment, the sensor and reference electrodes would be circular gold pads on the order of 100-500 uM in diameter, separated by a center-to center distance equal to twice their diameter. Each electrode would be addressed by separate electrical interconnects. The application of electrical signals to the sensor electrodes can be accomplished using standard commercially available AC and DC voltage sources. Detection of faradaic electrical signals

30   from the sensor electrodes can be accomplished easily using standard commercially available data acquisition boards mounted within and controlled by a microcomputer. Specifically, the raw sensor current signals would need to be amplified, and then converted to a voltage and analyzed via a high resolution (*i.e.*, 16 bit) analog to digital converter (ADC). It is possible to reduce the signal background and to increase the signal to noise

ratio (SNR) by using the common technique of phase-sensitive detection. In this detection method, an alternating current (AC) bias voltage (at a frequency between, for example, 100 to 1000 Hz) is superposed on top of the DC linear voltage ramp applied to the sensor electrodes. The frequency of the applied bias voltage is called the fundamental frequency. It

5   can be shown that the sensor response signal contains multiple frequency components, including the fundamental frequency and its harmonics (integral multiples of the fundamental frequency). It can further be shown that the nth harmonic signal is proportional to the nth derivative of the signal. Detecting these derivative signals (by means of a lock-in amplifier) minimizes the effects of constant or sloping backgrounds, and can enhance

10   sensitivity by increasing the signal to noise ratio and allowing the separation of closely spaced signal peaks. It should be noted that digital, computer-controlled AC and DC voltage sources (*i.e.*, digital to analog converters, DACs), current preamplifiers, analog to digital converters (ADCs), and lock-in amplifiers are all available as integrated signal generation/acquisition boards that can be mounted within and controlled by a single

15   microcomputer.

In a preferred embodiment, an integrated nucleic acid sensor molecule system with electrochemical detection would include the following elements one, a independently addressable multielement electrode array with immobilized surface layer composed of conductive species separated by insulating species and sensors; two, optical nucleic acid

20   sensor molecules immobilized on the electrode array; three, an oligonucleotide substrate/signaling probe which ligates with the nucleic acid sensor molecule in the presence of the appropriate target; four, an automated or semi-automated microfluidic reagent and sample delivery system; and five, a reader instrument/data acquisition system consisting of a microcomputer controlling the appropriate voltage sources, current and lock-

25   in amplifiers, data acquisition boards, and software interface for instrument control and data collection.

In another embodiment, the change in activity of the nucleic acid sensor molecule can be detected by watching the change in fluorescence of a nucleic acid sensor molecule when it is immobilized on a chip. A ligase can be attached to a chip and its ligase activity

30   monitored. Ligase ERK nucleic acid sensor molecules, labeled with one fluorophore, *e.g.*, Cy3 is attached via an amino modification to an aldehyde chip. The initial Cy3 fluorescence indicates the efficiency of immobilization of the nucleic acid sensor molecules. Next, the chip is exposed to a substrate labeled with a second fluorophore, *e.g.*, Cy5, with or without the ERK protein target. In the presence of ERK the nucleic acid sensor molecule

ligates the substrate to itself, and becomes Cy5-labeled. Without ERK, the ligation does not occur.

The use of a labeled effector oligonucleotide does not change the rate of ligation of the nucleic acid sensor molecule whether ERK is present or not. When using nucleic acid

5   sensor molecules in the context of a chip based system, in one embodiment, an effector oligonucleotide is used to attach the nucleic acid sensor molecule to the chip. Whether one uses effector oligonucleotide or not, the TaqMan (real-time PCR)traces obtained in the presence or absence of target (ERK) for ERK dependent ligases are identical. The presence or absence of this effector oligonucleotide does not affect the activity of the nucleic acid

10   sensor molecule.

In another embodiment, a hammerhead nucleic acid sensor molecule could be used to measure the concentration of an analyte through the use of fluorescence. Figure 82 shows how many nucleic acid sensor molecules with different effector molecules and/or analytes could be integrated onto one chip to study the concentration of many molecules at

15   once.

Any optical method known in the art, in addition to those described above can be used in the detection and/or quantification of all targets of interest in all sensor formats, in both biological and nonbiological media. These targets include, e.g., those listed in Table 1, below.

20   Any other detection method can also be used in the detection and/or quantification of targets. For example, radioactive labels could be used, including ^{32}P, ^{33}P, ^{14}C, ^{3}H, or ^{125}I. Also enzymatic labels can be used including horse radish peroxidase or alkaline phosphatase. The detection method could also involve the use of a capture tag for the bound nucleic acid sensor molecule.

25

4.   **Methods of Using Nucleic Acid Sensor Molecules**

A.   **Diagnostic Assays**

The nucleic acid sensor molecules according to the invention can be used to detect

30   virtually any target molecule. In one embodiment, the target molecule is a target molecule associated with a pathological condition and detection of changes in the optical properties of the nucleic acid sensor molecules of the biosensor provides a means of diagnosing the condition. Target molecules which are contemplated within the scope include, e.g. proteins, modified forms of proteins, metabolites, organic molecules, and metal ions, as discussed

above. Because signal generation in this system is reversible, washing of the biosensor(s) in a suitable buffer will allow the biosensor(s) to be used multiple times, enhancing the reproducibility of the any diagnostic assay since the same reagents can be used over and over. Suitable wash buffers include, *e.g.*, binding buffer without target or, for faster

5   washing, a high salt buffer or other denaturing conditions, followed by re-equilibration with binding buffer.

Re-use of the biosensor is enhanced by selecting optimal fluorophores. For example, Alexa Fluor 488, produced by Molecular Probes, has similar optical characteristics compared to fluorescein, but has a much longer lifetime. However, in one embodiment, a

10   site recognized by a nuclease is engineered proximal to the signal generating site, and sequences comprising signaling moieties are removed from the biosensor and replaced by new sequences, as needed.

Profiling Biosensors for Use in Diagnostic Assays

In one embodiment, the expression pattern of a plurality of target molecules is

15   determined to obtain a profile of target molecules associated with a trait in an individual to determine an expression pattern which is diagnostic of that trait. In this embodiment, combinations of biosensors targeted to individual target molecules are selected until a signature optical profile is determined which is characteristic of a trait. Traits include, *e.g.*, a disease, a genetic alteration, a combination of genetic alterations (*e.g.*, a polygenic

20   disorder), a physiological reaction to an environmental condition, or a wild type state (*e.g.*, of an organism or of an organ system). The target molecules which generate the signature optical profile are identified (based on the type of biosensors used) as signature target molecules. The expression of the signature target molecules can thereafter be determined to identify the presence of the trait in a patient.

25   The expression of the target molecules can be identified using any molecular detection system known in the art; however, in a preferred embodiment, the detection system comprises optical nucleic acid sensor molecules and the trait is identified by detecting the signature optical profile. In one embodiment, data relating to the signature optical profile is stored in the memory of a computer. Signature optical profiles can be

30   generated for individual patients or can be generated for populations of individuals. In the latter embodiment, data relating to a composite signature profile (*e.g.*, comprising normalized data) is stored in the memory of a computer or in a computer program product.

Because the biological function of the target molecules does not need to be known, biosensors according to the invention can be generated which are diagnostic of diseases/traits whose biological basis is not yet known or are the result of complex polygenic interactions and/or of environmental influences. In one embodiment, nucleic acid

5   sensor molecules are identified which are activatable by synthetic polypeptides obtained from putative open reading frames identified in the human genome project and/or in other sequencing efforts. Combinations of these activatable nucleic acid sensor molecules (along with activatable nucleic acid sensor molecules specific for target molecules with known functions) are identified which generate a diagnostic optical signal, and signature target

10   molecules are in turn identified which are linked to a particular trait, allowing a biological activity to be associated with a previously uncharacterized molecule.

Data relating to signature target molecules or to the optical signals generated upon activation of nucleic acid sensor molecules upon binding to signature target molecules is stored in a database, which can include further information such as sequence information or

15   chemical structure information relating to the signature target molecule. A signature profile relating to a particular trait is generated based on normalized data from a plurality of tests. In one embodiment, a signature profile is obtained by determining any or all of the level, chemical structure, or activity, of signature target molecules associated with a disease in samples from a population of healthy individuals to determine a signature profile

20   corresponding to a healthy state. In a further embodiment, signature profiles are obtained using data from subsets of populations which are divided into groups based on sex, age, exposure to environmental factors, ethnic background, and family history of a disease.

B.   Drug Discovery

25   Generally, methods of drug discovery comprise steps of 1) identifying target(s) molecules associated with a disease; 2) validating target molecules (e.g., mimicking the disease in an animal or cellular model); 3) developing assays to identify lead compounds which affect that target (e.g., such as using libraries to assay the ability of a compound to bind to the target); 4) prioritizing and modifying lead compounds identified through

30   biochemical and cellular testing; 5) testing in animal models; and 6) testing in humans (clinical trials). Through the power of genomics and combinatorial chemistry, large numbers of lead compounds can be identified in high throughput assays (step 3); however, a bottleneck occurs at step 4 because of the lack of efficient ways to prioritize and optimize lead compounds and to identify those which actually offer potential for clinical trials. The

target activatable nucleic acid sensor molecules according to the present invention offer a way to solve this problem by providing reagents which can be used at each step of the drug development process. Most importantly, the target activatable nucleic acid sensor molecules according to the present invention offer a way to correlate biochemical data, from *in vitro*

5   biochemistry and cellular assays, with the effect of a drug on physiological response from a biological assay.

In one embodiment of invention, a method for identifying a drug compound is provided, comprising identifying a profile of target molecules associated with a disease trait in a patient, administering a candidate compound to the patient, and monitoring changes in

10   the profile. In another embodiment, the monitored profile is compared with a profile of a healthy patient or population of healthy patients, and a compound which generates a profile which is substantially similar to the profile of target molecules in the healthy patient(s) (based on routine statistical testing) is identified as a drug. In a further embodiment, both the profiling and the drug identification step is performed using at least one nucleic acid

15   sensor molecule whose properties change upon binding to a target molecule.

In a further embodiment, a method for identifying a drug compound comprises identifying a plurality of pathway target molecules, each belonging to a pathway, monitoring the level, chemical structure, and/or activity of pathway target molecules in a patient having a disease trait, administering a candidate compound to the patient, and

20   monitoring changes in the level, chemical structure, and/or activity of the pathway target molecules. In another embodiment, the monitored level, chemical structure, and/or activity of the pathway target molecules is compared to the level, chemical structure, and/or activity of pathway target molecules in a wild type patient or patients. In a further embodiment, both profiling and the identification of drug compounds is performed using at least one nucleic

25   acid sensor molecule whose properties change upon binding to a pathway target molecule.

Properties according to this aspect include, *e.g.*, optical properties, change in sequence, chemical structure, catalytic activity, and/or molecular weight. In a preferred embodiment, sensor molecules are target activated optical nucleic acid sensor molecules.

i.   <u>Nucleic acid sensor molecules for Use in Identifying Lead Compounds</u>

30   In one embodiment, biosensors activatable by signature target molecules, identified as described above, are provided and are validated by testing against multiple patient samples *in vitro* to verify that the optical signal generated by these molecules is diagnostic of a particular disease. Validation can also be performed *ex vivo, e.g.*, in cell culture, (using

microscope-based detection systems and other optical systems as described in U.S. Patent Number 5,843,658, U.S. Patent Number 5,776,782, U.S. Patent Number 5,648,269, and U.S. Patent Number 5,585,245) and/or *in vivo*, for example, by providing a profile biosensor in communication with an optical fiber.

5   The incorporation of biosensors into fiber optic waveguides is known in the art (see, *e.g.*, U.S. Patent Number 4,577,109, U.S. Patent Number 5,037,615, U.S. Patent Number 4,929,561, U.S. Patent Number 4,822,746, and U.S. Patent Number 4,762,799). The selection of fluorescent energy transfer molecules for *in vivo* use is described in EP-A 649848, for example. In this embodiment, nucleic-acid based biosensors are introduced into

10   the body by any suitable medical access device, such as an endoscope or a catheter. The optical fiber is provided within a working lumen of the access device and is in communication with an optical imaging system.

           In one embodiment, the same methods which are used to validate the diagnostic value of particular sets of target molecule/nucleic acid sensor molecule combinations are

15   used to identify lead compounds which can function as drugs. Thus, in one embodiment, the effects of a compound on target dependent optical signaling is monitored to identify changes in a signature profile arising as a result of treatment with a candidate compound.

           In one embodiment, samples from a treated patient are tested *in vitro*; however, samples can also be tested *ex vivo* or *in vivo*. When the diagnostic profile identified by the

20   biosensor changes from a profile which is a signature of a disease to one which is substantially similar to the signature of a wild type state (*e.g.*, as determined using routine statistical tests), the lead compound is identified as a drug. Target molecules which activate the biosensor can comprise molecules with characterized activity and/or molecules with uncharacterized activity. Because large number of target molecules can be monitored

25   simultaneously, the method provides a way to assess the affects of compounds on multiple drug targets simultaneously, allowing identification of the most sensitive drug targets associated with a particular trait (*e.g.*, a disease or a genetic alteration).

           Examples of suitable target molecules include, *e.g.*, nuclear hormone receptor (NHR) polypeptides; G-coupled protein receptor (GPCR) polypeptides, phosphodiesterase

30   (PDE), and protein kinases.

           NHR polypeptides

Included in the invention are methods of identifying nucleic acid sensor molecules for detection of conformational isoforms of nuclear hormone receptors, as well as the nucleic acid sensor molecules identified by the methods described herein.

Nuclear hormone receptors (NHRs) act as ligand-inducible transcription factors by
5   directly interacting as monomers, homodimers, or heterodimers in complex with DNA response elements of target genes. The activation of these transcription regulators is induced by the change in conformation of the NHR upon complex formation with ligand.

Provided are methods for generating unique NASMs for each NHR ligand binding domain (LBD). The NASMs described herein can include, *e.g.*, those derived from the
10   hammerhead, hairpin, L1 ligase or group1 intron ribozymes and the like, any of which transduce molecular recognition into a detectable signal.

Also provided is a direct mechanistic assay for the action of small molecule ligand-agonism, -antagonism and partial antagonism of members of the NHR family. The mechanistic assays function in both *in vitro* biochemical as well as with *in vitro* cell-based
15   settings. In the *in vitro* assay setting, the nucleic acid sensor molecules are designed to recognize one conformational isomer of the NHR. In one embodiment, the nucleic acid sensor molecule recognizes the unique conformation that exists for the agonist bound form of a hormone receptor; such as that observed for the estrogen receptor ligand binding domain ER-LBD when bound to estrogen (Shiau AK, Barstad D, Loria PM, Cheng L,
20   Kushner PJ, Agard DA, Greene GL. Cell. 1998;95(7):927-37) and then produces a detectable signal, such as release of fluorescently labeled oligonucleotide, radiolabeled oligonucleotide, or reveals a change in nucleic acid sensor molecule conformation driven by ligand binding through a change in fluorescence or the like. Hence, in this embodiment, the nucleic acid sensor molecule transduces molecular recognition of the ER-LBD-estrogen
25   agonist complex into a detectable signal. The level of the signal is then used to quantify the amount of ER-LBD-estrogen agonist complex present in solution. In another embodiment, the ER-LBD-estrogen specific nucleic acid sensor molecule is used as a screening tool in assays designed to detect inhibitors of ER-LBD-estrogen complex formation. These screening tools can be used to determine the inhibition potency of compounds in *in vitro*
30   biochemical assays or in *in vitro* cell-based assays. Inhibitors of estrogen binding to ER-LBD are useful as anti-proliferative agents for treatment of breast cancers (*e.g.*, tamoxifen) and other estrogen dependent diseases. In another embodiment, nucleic acid sensor molecules are introduced into cell lines by known methods of electroporation, transfection or coupling to peptide translocating agents such as tat or antennapedia peptides. In another

embodiment, the ER-LBD-estrogen complex specific nucleic acid sensor molecule is an allosteric intron imbedded in a reporter gene such as GFP or luciferase or the like. When the intron derived nucleic acid sensor molecule is inserted into the reporter gene it renders reporter gene expression effector dependent. Thus, in one embodiment functional GFP

5   protein is expressed only when the ER-LBD-estrogen complex is present in the cell, and inhibitors of ER-LBD-estrogen complex formation thus block functional GFP protein expression in appropriate mammalian, such as MCF7 or T47D, yeast or bacterial cell lines. In a further embodiment, the MCF7 or T47D tumor cell lines transfected with GFP-ER-LBD-estrogen nucleic acid sensor molecule sensitive construct are used to form tumor

10   xenografts in nude mice. Thus, the transfected tumor xenograft cell lines can be used to form tumors in mice which are not only estrogen dependent but also regulate reporter gene expression in ER-LBD-estrogen dependent manner. These cell lines and tumor models are used to discover inhibitors of ER-LBD-estrogen complex formation *in vivo*.

NHR ligand binding domains bind antagonists, forming additional conformational

15   isomers. When antagonists are bound to the receptor a new conformer results such as that observed upon tamoxifen binding to the estrogen receptor to form a stable ER-LBD-tamoxifen complex (Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. Cell. 1998 Dec 23;95(7):927-37). Accordingly, the invention includes use of an ER-LBD-tamoxifen specific nucleic acid sensor molecule that is used to detect the levels

20   of antagonist specific complex in both *in vitro* biochemical, cell-based and, in *in vivo* assays as described above.

Nucleic acid sensor molecules can be developed that are specific for the ligand binding domains of all NHRs. In addition, it should be clear that nucleic acid sensor molecules for agonist, antagonist, dimeric or multimeric forms of NHR LBDs can be used

25   to screen for inhibitors of LBD function and therefore for inhibitors of NHR dependent transcriptional activation or repression. It should be clear to one skilled in the art that nucleic acid sensor molecules specific for individual LBD complexes can be used to screen for agents that modify NHR function in *in vitro* and in *in vivo* assays.

NHRs are multidomain proteins containing a variable NH$_2$-terminal region (A/B), a

30   conserved DNA binding domain (DBD) or region (C), a linker region (D), and a conserved region (E) that contains the ligand binding domain (LBD). NHRs also contain regions required for transcriptional activation, of particular interest is the region AF-2 which is located in the COOH-terminus and whose function is strictly ligand dependent. Provided herein is a method for generating unique nucleic acid sensor molecules to each of the 63

known human NHR LBDs. In addition, methods are described that enable the generation of nucleic acid sensor molecules capable of recognizing the activated state of the NHR by selection for nucleic acid sensor molecules geometries which signal the presence of either the activated or inactivated conformation (NHR with bound ligand), but whose signaling

5   action is quiescent in the presence of other forms of the NHR.

The nucleic acid sensor molecules allow the direct, simultaneous, and rapid detection of the activation states of all NHRs. This tool can be used in *in vitro* assays for receptor activation with agonists and antagonists, and can be used to generate cell lines and animal models that report on the activation state of such receptors in a biological setting and

10   as a function of drug or drug lead.

GPCR Nucleic acid sensor molecules .,

Also provided by the invention are nucleic acid sensor molecules for detection of conformational isoforms of G-protein coupled receptors.

G-protein coupled receptors (GPCRs) play fundamental roles in regulating the

15   activity of virtually every cell. Upon binding of extracellular ligands, GPCRs interact with a specific subset of heterotrimeric G-proteins that can then, in their activated forms, inhibit or activate various effector enzymes and/or ion channels. Molecular cloning studies have identified multiple human GPCRs, and have identified the ligands for many of these.

GPCRs include three domains: an extracellular N-terminus, a central domain of

20   seven trans-membrane helices connected by unstructured loops, and a cytoplasmic C-terminus. Activation of GPCRs is induced by ligand binding, which causes a conformational change in the receptor transmitting a signal across the plasma membrane to intracellular members of a signaling pathway. The methods described herein allow for generating unique nucleic acid sensor molecules to any GPCR. In addition, methods are

25   described that enable the generation of nucleic acid sensor molecules capable of recognizing the activated state of the GPCR by selecting for nucleic acid sensor molecule geometries which signal the presence or absense of the activated conformation of the receptor through recognition of one or all of the mobile domains, but whose signaling action is quiescent in the presence of other forms of the GPCR. The biosensors described herein include nucleic

30   acid sensor molecules such as allosetric ribozymes (AR), including those derived from the hammerhead, hairpin, L1 ligase, or group 1 intron ribozymes and the like. Nucleic acid sensor molecules may also be derived from aptamer beacons or signaling aptamers which transduce molecular recognition into a detectible signal.

Upon the activation by an extracellular ligand or stimuli, G-protein coupled receptor(GPCR) polypeptides activate intracellular Gα-protein. A single GPCR can activate a number of Gα-proteins. For example, adrenergic receptors activate Gi, which inhibit adenylyl cyclases, Gs, which stimulate adenylyl cyclases, and Gq, which regulate

5   cellular Calcium ion level (Wenzel-Seifert and Seifert 2000). Thus, it is highly desirable to distinguish which class of Gα-proteins are activated through the GPCR of interest in cells.

The initial drug screening of the GPCRs is normally performed by competition assay with radiolabeled ligands. For a cell based GPCR assay, incorporation of radiolabeled GTP can be measured to detect the coupling of Gα-protein and GPCR, however this assay does

10   not distinguish the type of Gα-proteins involved. The assays for the effect on individual effectors, such as the Ca^{++} flow or cellular cAMP level, are also commonly used, but only one downstream signal can be measured at a time using these assays.

Upon activation, a Gα-protein undergoes significant conformational change which results in release of GDP and association with GTP (Coleman and Sprang 1998). The Gα-

15   protein also dissociates from its βγ-subunits. This activated form of Gα-protein then becomes capable of interacting with its effector (Li, Sternweis et al. 1998). The well-characterized conformation change takes place in three switches; switch I (residues 177-187 in Giα1), switch II (residues 199-219 in Giα1), and switch III (residues 231-242 in Giα1). The sequences and the conformational changes in these switches are well conserved among

20   Gα-proteins.

Ras is a member of the small GTPase protein family, which shares significant similarity with other family members. GTP-bound ras and GDP-bound ras can be distinguished by the RBD (ras binding domain) of Raf-1 (Taylor, Resnick et al. 2001). The activated state of Raf-1 can be identified by RalRDS (Franke, Akkerman et al. 1997). This

25   indicates significant change in the surface of the protein, and the effector binding surface are only available for interaction in GTP complex form.

The invention provides methods for selecting nucleic acid sensor molecules which recognize the conformational change upon GTP binding and/or specifically interact with newly exposed G-protein effector binding sites upon the activation. Class-specific activated

30   Gα-protein nucleic acid sensor molecules recognize the activated Gα-proteins or its effector binding site, which allow us to interpret the multiple type of downstream signal affect by the GPCR. It can used in both *in vitro* HTS (high throughput screening) and cell-based HTS.

Also described is a method for developing a direct mechanistic assay of the action of small molecule ligand-agonism, -antagonism, and partial antagonism of members of the GPCR family. The mechanistic assays function in both *in vitro* biochemical and *in vitro* cell-based settings. In the *in vitro* assay setting, the nucleic acid sensor molecules are designed to recognize one conformational isomer of the GPCR.

5

In one embodiment, the nucleic acid sensor molecule recognizes the unique conformation that exists for the activated state when in complex with ligand, *e.g.*, such as that observed for the beta-2 adrenergic receptor when in complex with the artificial ligand isoproterenol (Ghanouni et al., PNAS USA, 98:5997-6002(2001)) and then produces a detectable signal, *e.g.*, by release of a fluorescently labeled oligonucleotide, release of a radiolabeled oligonucleotide, or a change in conformation of the NASM driven by ligand binding through a change in fluorescence or the like. Hence, in this embodiment, the nucleic acid sensor molecule transduces molecular recognition of the beta-2 adrenergic receptor – in complex with epinephrine, norepinephrine or an artificial ligand such as isoproterenol into a detectible signal. The level of the signal is then used to quantify the amount of beta-2 adrenergic receptor-agonist complex present in solution.

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In another embodiment, the beta-2 adrenergic-agonist nucleic acid sensor molecule is used as a screening tool in assays designed to detect agonists of the beta-2 adrenergic receptor. These screening tools can be used to determine the activation potency of compounds in *in vitro* biochemical assays or in *in vitro* cell-based assays. Agonists of the beta-2 receptor are useful in the treatment of asthma (Robinson, et al. Lancet 357:2007-2011(2001)). In another embodiment, nucleic acid sensor molecules are introduced into cell lines by known methods of electroporation, transfection, or coupling to peptide translocating agents such as tat or antennapedia peptides.

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In another embodiment, the beta-2 adrenergic receptor-agonist complex specific nucleic acid sensor molecule is an allosteric intron imbedded in a reporter gene such as GFP or luciferase or the like. When the intron-derived reporter is inserted into the reporter gene it renders reporter gene expression effector dependent. Thus, in one embodiment functional GFP protein is expressed only when the beta-2 adrenergic receptor-agonist complex is present in the cell, and inhibitors of beta-2 adrenergic receptor-agonist complex formation block functional GFP protein expression in appropriate cells such as mammalian human peripheral blood leukocytes, yeast, insect, or bacterial cell lines.

30

In a further embodiment, Chinese hamster fibroblasts, which do not express beta-adrenergic receptors (Sheppard, et al., PNAS USA 80:233-236(1983)), are transfected with

both the nucleic acid sensor molecule and the gene coding for the beta-adrenergic receptor under a constitutive promoter, and are used to create a model cell line suitable for HTS screening of candidate beta-2 agonists. Furthermore, cells can be caused to express known allelic variants, such as gln27-to-glu associated with obesity (Large, et al., J Clin. Invest

5   100:3005-3013), to create cells lines which model specific disease states. Furthermore, chimeric mice can be created by "knock-in" (Monroe et al., Immunity 11:201-212(1999)) which will express the nucleic acid sensor molecule in every cell as the result of blastocyst fusion (Chen et al., PNAS USA 90:4528-4532(1993)), and used for pharmokinetic or bioavailability studies in which the GPCR activation states of various tissues in the

10   organism are of concern.

GPCRs bind antagonists, which cause the GPCRs to become resistant to conformational changes, or result in conformations not susceptible to activation, or blockade the ligand binding domain from interaction with the appropriate ligand and thus prevent activation of the GPCR, such as the beta-2 adrenergic receptor antagonist

15   butoxamine (Horinouchi et al., Pharmacology 62:98-102(2001)). Hence, the invention also provides a method for using a nucleic acid sensor molecule to detect conformers which result from binding of GPCRs to antagonists. Furthermore, when the cell line described above is transfected with a mutant variants of GPCRs which spontaneously adopt the active conformation, such as lys272-to-ala (Pei, et al., PNAS USA 91:2699-2702(1994) and

20   references therein) the nucleic acid sensor molecule can be employed in a screen for compounds which are beta-2 antagonists (Ramsay et al, Br J Pharmacol 133:315-323(2001)). Antagonists of the beta-2 receptor are useful in the treatment of cardiovascular diseases (Nagatomo, et al., Cardiovasc Drug Rev 19:9-24(2001)). The invention accordingly provides a method for using a Beta-2 adrenergic receptor – butoxamine

25   complex-specific nucleic acid sensor molecule that is used to detect the levels of an antagonist specific complex in both *in vitro* biochemical, cell-based, and *in vivo* assays as described above.

Nucleic acid sensor molecules can also be developed that are specific for the occupancy state of the ligand-binding domains of all GPCRs. In addition, nucleic acid

30   sensor molecules for the agonist, antagonist, dimeric, or multimeric forms of all GPCRs can be used to screen for inhibitors or activators of GPCR function and therefore for inhibitors or activators of GPCR-dependent cell signaling pathways. Nucleic acid sensor molecules specific for individual GPCR complexes can be additionally used to screen for agents that modify GPCRs in *in vitro* and *in vivo* assays.

Phosphodiesterase-specific nucleic acid sensor molecules

Multiple classes of phosphodiesterases (PDEs) have been identified in humans. These enzymes catalyze a reaction that converts second messenger cAMP and cGMP into
5   5'-AMP and 5'-GMP. Different class of PDEs have different substrate specificity as well as different physiological function. For example, PDE4s are specific for cAMP and PDE5 are specific for cGMP.

The invention provides multiple classes of PDE nucleic acid sensor molecules. The first class of nucleic acid sensor molecules can distinguish cAMP vs. 5'AMP (cGMP vs
10   5'GMP) (Koizumi, Kerr et al. 1999) (Koizumi, Soukup et al. 1999). The second class of nucleic acid sensor molecule binds to the active site of PDE in a class specific manner and inhibits PDE catalytic activity. This class of nucleic acid sensor molecule can be raised using PDEs in the presence and absence of high affinity known inhibitors (*e.g.*, Ropalim for PDE4). The third class of nucleic acid sensor molecule recognizes PDE in a class-specific
15   (*e.g.*, PDE1-11) or subclass-specific (PDE4A-D) manner.

Protein kinase-specific nucleic acid sensor molecules

The invention also provides nucleic acid sensor molecules raised against protein kinases. In one embodiment, the invention provides nucleic acid sensor molecules that are modulated by the phosphorylation state in a given peptide sequence. Alternatively, native
20   proteins can be used with different phosphorylation states in order to raise nucleic acid sensor molecules that can distinguish the different phosphorylation states. For example, ERK1/2 and phosphorylated ERK1/2 can be distinguished by specific nucleic acid sensor molecules (Seiwert, Stines Nahreini et al. 2000). The nucleic acid sensor molecule also can be a competitive inhibitor for a kinase by binding at ATP or substrate binding sites.
25   Alternatively, an ADP-dependent nucleic acid sensor molecule can be obtained at lower pH. These nucleic acid sensor molecules can be used to detect the production of ADP.

ii.   Pathway Profiling Biosensors

As shown in Figure 7, physiological function is modulated by complex pathways,
30   each of which may have multiple overlapping and intersecting steps. Furthermore, the proteins involved in these pathways are highly homologous and can have overlapping substrates and drug specificities. Using current techniques, it is possible only to monitor the

response of single elements of a pathway. These techniques are inadequate to understand the mechanism of drug interactions. For example, a particular drug found to have a particular *in vitro* activity against a single target in biochemical assays might interact with other proteins in the same pathway, or in other unrelated pathways. Consequently,

5 physiological function is often uncorrelated with the results of biochemical assays of a single target.

The nucleic acid sensor molecules according to the invention provide reagents to simultaneously quantify the level and chemical state of all components in a molecular pathway As used herein, "pathway target molecules" are target molecules involved in the

10 same pathway and whose accumulation and/or activity is dependent on other pathway target molecules, or whose accumulation and/or activity affects the accumulation and/or activity of other pathway target molecules. Pathway target molecules according to the invention include, *e.g.*, proteins, such as enzymes, modified forms of proteins, such as phosphorylated, sulfated, ribosylated proteins, methylated proteins (Arg, Asp; N, S or O

15 directed), prenylated proteins (such as by farnesyl, geranylgeranyl, and other types of prenylation) acetylated or acylated proteins, cleaved or clipped proteins, bound or unbound forms of proteins, allelic variants of a protein (*e.g.*, proteins differing from each other by single amino acid changes in a protein), as well as substrates, intermediates, and products of enzymes (including both protein and non-protein molecules).

20 In another embodiment, diagnostic pathway target molecules are identified by pre-selecting a plurality of nucleic acid sensor molecules activatable by pathway-specific target molecules. In one embodiment, a profiling biosensor is provided comprising at least one nucleic acid sensor molecule specific for every molecular species within a pathway (*e.g.*, a signaling pathway), to generate a biosensor which can monitor the levels, chemical

25 structure, and/or activity of every molecular species in the pathway.

Because of the finite number of target molecules (as determined from data obtained from the Human Genome Project) and the high throughput of the biosensors of the instant invention (greater than 10,000 target molecules can be monitored simultaneously), the profiling biosensors of the instant invention make it feasible to evaluate the response of all

30 the components of a pathway to a drug compound simultaneously.

In one embodiment, a profiling biosensor reactive to the components of an entire pathway, is contacted with a sample from a patient having a disease, and an optical signal corresponding to a disease state is determined to identify diagnostic pathway target molecules which are diagnostic of that disease. Samples from a plurality of patients are

obtained and tested using the profiling biosensor to identify a pathway profile that is
diagnostic of the disease, the pathway profile comprising normalized data relating to any or
all of the level, structure, and activity, of the signature pathway molecules. A pathway
profile corresponding to a wild type state is determined by testing the profiling biosensor

5   molecules against samples from a population of healthy patients, or subsets of populations
of healthy patients. In one embodiment, data relating to the optical signals generated by
nucleic acid sensor molecules activated by the diagnostic pathway target molecules is stored
within the memory of a computer or within a computer program product.

        The pathway profiles can be used in diagnostic testing as discussed above. In one

10   embodiment, a profiling biosensor is used in which the pathway is one which is known or
suspected of being disrupted in patients having a particular trait (*e.g.*, having a particular
disease or genetic alteration(s)). For example, in one embodiment, one profiling biosensor
used to evaluate samples from a patient with cardiovascular disease is a cholesterol
metabolism pathway profiling biosensor. However, random combinations of profiling

15   biosensors can be used to assess the physiological state of a patient, to identify diagnostic
pathway profiles which are diagnostic of diseases whose molecular basis has not yet been
identified or characterized.

        In one embodiment, profiling biosensors according to the invention are used to
assess the affect of a candidate drug on any or all of the level, chemical structure, or activity

20   of diagnostic pathway target molecules to generate a drug treatment pathway profile. In this
embodiment, a profiling biosensor is contacted with a sample from a cell or physiological
system (*e.g.*, a group of cells, a tissue system, an organ system, or a patient), and changes in
optical signals are obtained which are correlated to any, or all of, the level, chemical
structure, or activity of a particular pathway target molecule by relating the optical signal

25   obtained to the address of the nucleic acid sensor molecule, as described above. In one
embodiment, a drug treatment profile which is substantially similar to a diagnostic pathway
profile obtained from a healthy population of patients (as determined by observing no
significant differences in the profile by routine statistical testing, to within 95% confidence
levels) is used to identify a candidate drug as one which is suitable for further testing. The

30   profile produced by such a drug is used to produce an effective drug treatment profile,
against which other candidate drugs can be compared.

        In another embodiment, a candidate drug is tested against a plurality of profiling
biosensors including the one which will generate a diagnostic signature profile, to identify
drugs which produce an effective drug treatment profile without effecting significant

changes in other pathway profiles. In this embodiment, the systemic effects of a candidate drug can be predicted.

In further embodiments, it is desirable to use a biosensor representing less than an entire pathway. In one embodiment, a biosensor is provided comprising nucleic acid sensor molecules diagnostic pathway target molecules. In a further embodiment, a biosensor is provided which comprises nucleic acid sensor molecules necessary to evaluate particular components of a pathway suspected of being involved in a disease. For example, compounds being screened to identify candidate drugs that affect diseases relating to defective DNA repair can be tested against a pathway biosensor comprising only S phase cell cycle target molecule reactive nucleic acid sensor molecule.

Exemplary pathway target molecules include various proteins and modified form thereof. Modifications include post-translational modifications such as phosphorylation, prenylation, glycosylation, methionine removal, N-acetylation, acylation, acylation of cysteines, myristoylation, alkylation, ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycoslylation (*e.g.*, of hemoglobin), deamination of glutamine and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation, and lipidation. Examples of such proteins include ERK and phosphorylated ERK, CDK and phosphorylated CDK, modified cyclin A, cyclin D, cyclin E, k-Ras, h-Ras, Rho A, MEK-1, MEKK-1, Raf-1, Raf-A, JNK, PKA, ATK, PTEN, p53, P16, and INK4. Other proteins include those listed in Table 1 as well as post-translationally modified forms thereof.

Table 1

apoptotic pathways		Bcl, Bak, ICE proteases, Ich-l, CrmA, CPP32, APO-l/Fas, DR3, FADD containing proteins, perform, p55 tumor necrosis factor (TNF) receptor, NAIP. TAP, TRADD-TRAF2 and TRADD-FADD, TNF, D4-GDI, NF-kB, CPP32/apopain, CD40, IRF-i, p53, apoptin
blood clotting pathways		thrombin, fibrinogen, factor V, Factor VIII- FVa, FVIIIa, Factor XI, Factor Xia, Factors IX and X, thrombin receptor, thrombomodulin (TM), protein C (PC) to activated protein C (aPC). aPC, plasminogen activator inhibitor-i (PAT-i), tPA (tissue plasminogen activator)
calcium signaling pathways		calmodulin, calcineurin,
Cell cycle Pathway	G0	MPS, CYTOSTATIC FACTOR (CSF) (INCLUDING Mos)
	G1	mid GI phase: cdk4/cycin DI-3 and cdk6/cyclin DI-3 late G 1 phase: cdk2/cycin E others: p53, p21, p16, Rb, p2'l, E2F, Cdc25A, Cdc25B

	S	cyclin A/CDK2, cyclin B/Cdc2, SPA-i, Cdc25A, Cdc25B
	G2	G2/M transition phase: cdkl/cyclin B 1-3, cdkl/cyclin A, Cdc25A, Cdc25B, Cdc25C. PIN!, Chki, Myt 1, Wee 1
	M	Cdc2/cyclin B, P1k, Cdc25C,
Cholesterol metabolism pathway		LDL, LDL-receptor, VLDL, HDL, cholesterol acyltransferase, apoprotein E, Cholesteryl esters, ApoA-I and A-II, HMGCoA reductase, cholesterol
Flt-3 pathway		flt-3 pathway flt-3, GRP-2, SHP-2, SHIP, She
JAK/STATS signaling pathway		Jakl, Jak2, IL-2, IL-4 and IL-7, Jak3, Ptk-2, Tyk2, EPO, GH, prolactin, IL-3, GM-CSF, G-CSF, IFN gamma, LW, OSM, IL-12 and IL-6, IFNR-alpha, IFNR- gamma , IL-2R beta , IL-6R, CNTFR, Stat 1 alpha, Stat 1 beta , and Stats2-6
MAP kinase signaling pathways		flt-3, ras, raf, Grb2, Erk-i, Erk-2, and Src, Erb2, gpl3O, MEK-1, MEK-2, hsp 90, JNK, p38, Sin!, Styi/Spcl, MKK's, MAPKAP kinase-2, JNK,SAPK
P53 pathway		bax, bid, caspases, cytochrome c
PI 3 kinase pathway		SHIP, Akt
ras activation pathways		p120-Ras GAP, neurofibromin, Gapl, Ra!-GDS, Rsbs 1, 2, and 4, Rinl, MEKK- 1, and phosphatidylinositol-3-OH kinase (P13K), ras
SIP signaling pathways		GRB2, SIP, ras, P1 3-kinase
SHC signaling pathways		trkA, trkb, NGF, BDNF, NT-4/5, trkC, fNT-3, Shc, PLC gamma 1, P1-3 kinase, SNT, ras, rafi, MEK and MAP kinase
TGF-13 signaling pathways		BMP, Smad 2, Smad4, activin, TGF
T-cell receptor complex		lck, fyn, CD4, CD8, T cell receptor proteins
MHC-I pathways		TAP proteins, LMP 2, LMP 7, gp 96, HSP 90, HSP 70

If desired, nucleic acid sensor molecules can be raised against particular amino acid sequences in the polypeptides. Some representative peptide regions are presented in Table 2.

5

Table 2

Sequence	Enzyme
L-R-A-S-L-G (SEQ ID NO:52)	PKA
A-A-K-I-Q-A-S-F-R-G-H-M-A-R-K-K (SEQ ID NO:53)	PKC
P-K-T-P-K-K-A-K-K-L(SEQ ID NO:54)	cdc2
E-P-P-L-S-Q-E-A-F-A-D-L-W-K-K(SEQ ID NO:55)	DNA-PK
D-D-D-E-E-S-I-T-R-R(SEQ ID NO:56)	CK-1
R-R-R-E-E-E-T-E-E-E (SEQ ID NO:57)	CK-2
K-K-A-L-R-R-Q-E-T-V-D-A-L (SEQ ID NO:58)	CaM KII
S-T-K-V-P-Q-T-P-L-H-T-S-R-V (SEQ ID NO:59)	P38
R-R-R-R-S-I-I-F-I (SEQ ID NO:60)	PKA
R-R-R-R-R-K-G-S-F-R-R-K-A (SEQ ID NO:61)	PKCα
R-K-L-K-R-K-G-S-F-R-R-K-A (SEQ ID NO:62)	PKCβ I, II
R-R-R-R-R-K-G-S-F-K-K-F-A (SEQ ID NO:63)	PKCγ
A-A-R-K-R-K-G-S-F-F-Y-G-G (SEQ ID NO:64)	PKCδ

Y-Y-X-K-R-K-M-S-F-F-E-F-D (SEQ ID NO:65)	PKCε
A-R-L-R-R-R-S-F-R-R-X-R (SEQ ID NO:66)	PKCη
R-R-F-K-R-Q-G-S-F-F-Y-F-F (SEQ ID NO:67)	PKCζ
A-A-L-V-R-Q-M-S-V-A-F-F-F (SEQ ID NO:68)	PKCμ
K-R-Q-Q-S-F-D-L-F (SEQ ID NO:69	CaM KII
F-R-M-M-S-F-F-L-F (SEQ ID NO:70)	Phosphorylase kinase
R-R-F-G-S-L-R-R-F (SEQ ID NO:71	SLK1
R-R-R-H-S-R-R-R-R (SEQ ID NO:72)	SRPK2
R-K-R-X-R-T-Y-S-F-G (SEQ ID NO:73)	AKT/PKB

In one embodiment, a profiling biosensor array is generated comprising target activatable nucleic acid sensor molecules which are activatable by components of a cell cycle pathway. In this embodiment, a cell cycle biosensor is generated comprising nucleic acid nucleic acid sensor molecules activatable by at least two members selected from the group consisting of: MPS, Cytostatic factor (CSF) (including Mos), cdk4, cyclins D1-3, cdk6, cdk2, cyclin E, p53, p21, p16, Rb, p27, E2F, cyclin A, cyclin B, cdk1, cyclin B1-3, Cdc2, SPA-1, and other biomolecules involved in cell cycle regulation.

In another embodiment, the cell cycle biosensor array generated is used to evaluate samples from patients suspected of having a disorder affecting cell proliferation (e.g., cancer) and a signature target molecule profile is determined which is diagnostic of this disorder. Changes in the signature target molecule profile upon treatment with a candidate compound are subsequently monitored by any or all of *in vitro, ex vivo*, and *in vivo* methods, as described above, to identify and/or validate lead compounds for use in cancer therapies.

In further embodiments, a cell cycle biosensor is provided comprising a plurality of locations, each location comprising a set of nucleic acid sensor molecules activatable by target molecules which identify a different portion of the cell cycle. Thus, in one embodiment, a cell cycle biosensor comprises at a first location, nucleic acid sensor molecules activatable by G0 specific target molecules (e.g., MPS, Cytostatic factor (CSF) (including Mos)), at a second location, nucleic acid sensor molecules activatable by G1 specific target molecules (cdk4, cyclin D1-3, cdk6, cdk2, cyclin E, p53, p21, p16, Rb, p27, E2F), at a third location, nucleic acid sensor molecules which are activatable by S specific target molecules (e.g., cyclin A/CDK2, cyclin B/Cdc2, SPA-), at a fourth location, nucleic acid sensor molecules activatable by G2 specific target molecules (e.g., cdk1, cyclin B 1-3, cyclin A), and at a fifth location, nucleic acid sensor molecules activatable by M specific

target molecules (*e.g.*, Cdc2, cyclin B). In this way the effects of diseases and/or drugs on specific phases of the cell cycle can be assessed.

Similarly, pathway specific biosensors can be generated for any of apoptotic pathways, blood clotting pathways, calcium regulation pathways, cholesterol metabolism pathways, the JAK/STATS signaling pathway, MAP kinase signaling pathways, p53 pathway, PI 3 kinase pathway, ras activation pathways, SIP signaling pathways, SHC signaling pathways, TGF-13 signaling pathways, T-cell receptor complex, and MHC-I pathways, using exemplary target molecules listed above, or other target molecule components of the respective pathways.

It should be apparent to those of ordinary skill in the art, that many other pathways exist whose components have been characterized and that target molecules within these pathways are also encompassed within the scope of the present invention (*e.g.*, including, but not limited to, phosphatase pathways, transcription factor pathways, hormone dependent pathways, as well as intermediary metabolism pathways, and developmental pathways). Further, additional pathways can be identified using the nucleic acid based biosensor profiling techniques discussed above (*e.g.*, identifying pathway molecules involved in the functioning of a wild type or diseased organ system, such as the cardiovascular system, central nervous system, digestive system, reproductive system, pulmonary system, skin system, and the like), and these also are encompassed within the scope of the invention.

Alternatively, or additionally, pathway specific molecules can be identified by other techniques known in the art (*see, e.g.*, U.S. Patent Number 6,087,477, U.S. Patent Number 6,054,558, U.S. Patent Number 6,048,709, and U.S. Patent Number 6,046, 165) and used to engineer additional pathway target activatable nucleic acid sensor molecules. Because there is a finite number of pathway target molecules in each pathway (constrained by the absolute number of gene products which have been identified) (see, *e.g.*, Drews, Science 287: 1960-1964), it is feasible using the target activatable nucleic acid sensor molecules to generate biosensors representative of an entire pathway.

In further embodiments, sets of profiling biosensors are used to monitor the expression/activity of target molecules representing complex systems. Thus, for example, the effect of target molecules on the cardiovascular system and pulmonary system can be monitored simultaneously. In one embodiment, an array representative of a plurality of systems in the human body is used in methods to assess the effects of drug compounds on multiple systems in the body.

iii. Using Pathway Nucleic Acid Sensor Molecules in Drug Optimization

The profiling nucleic acid sensor molecules according to the invention can be used
5   in every step of a drug optimization process, as shown in Figure 8, and are suitable reagents
for use in conventional high throughput screening systems making them extremely
adaptable for use alone, or in conjunction with, other drug development assays.

Step 1.   Drug Target Discovery or Drug Target Validation

As discussed above, profiling nucleic acid sensor molecules can be used to identify
10   signature target molecules which are correlatable to particular traits, such as disease.
Signature target molecules are drug targets whose levels, structure, and/or activity can be
used to evaluate the efficacy of compounds. A large number of signature drug targets, both
characterized and uncharacterized, can be identified simultaneously using a single profiling
biosensor according to one embodiment. In one embodiment, a profiling biosensor
15   recognizes and is independently activated by about 1-5,000 molecules. In another
embodiment, a profiling biosensor recognizes and is independently activated by about 500-
10,000 molecules, and in another embodiment, by greater than 10,000 molecules.

Step 2.   High Throughput Screening

In one embodiment, the drug targets identified in step 1 are evaluated in high
20   throughput screening assays, using either solution-based biosensors or substrate-based
biosensors, to characterize the biological activity of a drug target. For example, in one
embodiment, nucleic acid sensor molecules are used to assess levels of substrate, product
and intermediates produced by an enzyme in a wild type vs. a disease state, to identify other
components of a pathway that would be affected by a drug acting on that target (i.e.,
25   secondary drug targets). In another embodiment, the levels, structure, and/or activity of all
of the modified forms of a drug target, or the active and inactive forms of a drug target (e.g.,
a receptor) is determined in a wild type vs. a disease state, to further develop a diagnostic
profile of a diagnostic pathway target molecule and to evaluate changes of that profile in the
presence of a drug.

30   In a further embodiment, the same type of profiling biosensor used to identify a
diagnostic profile is contacted with samples from patients exposed to a compound. A
compound-treated sample which produces substantially similar levels, structure, and/or

activity of target and secondary drug targets in a sample from a healthy patient is used to identify a compound as a candidate drug. Because this testing is done in a high throughput format, a single dose of a candidate drug is evaluated in any given test.

5 **Step 3.** *In vitro* Biochemical Assays

In one embodiment, the nucleic acid sensor molecules used in step 2, are tested in an *in vitro* biochemical assay to determine compound potency. In this embodiment, a preliminary dosing effect is determined to identify the IC50 of candidate drug. In one embodiment, multiple biosensors of the type used in step 2 are contacted with samples from

10 patients exposed to different doses of the candidate drugs identified in step 2, to identify candidate drugs with the highest potency (*e.g.*, requiring the least amount of drug to generate a wild type profile or an effective drug profile.

Step 4. Cellular Assays

15 In one embodiment, nucleic acid sensor molecules are used in cellular assays where the effect of adding a compound on cell physiology is known and the researcher wants to determine that the drug is in fact acting through the drug target selected in steps 1-3. Here a candidate drug is added to a physiological system (*e.g.*, cell(s), tissue(s), organ(s), or a patient). Cells from the physiological system are lysed and the substrate or product of an

20 enzyme reaction is monitored using the nucleic acid sensor molecule either in an ELISA format or other solid support-based format (*e.g.*, a profiling array) or a solution phase format. In another embodiment, cell lysates are contacted with a profiling biosensor specific for a target or pathway of interest to determine the profile of target molecules in the lysed sample. The profile is then compared to the wild type profile and the disease profile to

25 determine if the drug is operating *in vivo* to restore a cell to its wild type state. Thus, the physiological effect of a candidate drug on a physiological system is correlated with the *in vivo* mechanism of action of the candidate drug.

In a preferred embodiment, pathway profiling arrays comprised of nucleic acid sensor molecules affixed to a solid support are used in cellular assays to determine the

30 selectivity of a compound for one target in a pathway relative to other candidate targets in a signal transduction pathway(s) or in another biochemical pathway(s). This data can be used to validate a drug lead or drug target.

In one embodiment, nucleic acid sensor molecules are expressed *in vivo* or intracellularly using plasmids, viruses or other extra-chromosomal DNA vectors and the

cellular nucleic acid sensor molecules are extracted and used to determine the activity of a drug or drug target. These cellular assays can also determine the selectivity of a compound for one target in a pathway relative to other candidate targets in a signal transduction pathway(s) or in another biochemical pathway(s). This data can be used to validate a drug

5   lead or drug target.

In vivo detection:

With (Amersham) SPA scintillant beads coupled to nucleic acid sensor molecules, one can look at cellular processes in situ in real time, by culturing cells directly onto a

10   microtiter plate and allowing uptake of scintillant beads and radioisotope by cells. One can then monitor biosynthesis, proliferation, drug uptake, cell motility, etc. via luminescence generated by beads in the presence of selected target.

Step 5.   **Medicinal Chemistry**

15   In one embodiment, drug-lead potency, specificity, and/or *in vivo* activity is optimized by an iterative repetition of any or all of steps 1-4. In one embodiment, steps 1-4 are repeated until the desired potency, selectivity and *in vivo* mechanism of action of a candidate drug is obtained. Potency can range from picomolar affinity to nanomolar affinity as measured by *in vitro* IC50 values. The desired selectivity of a drug candidate for its target

20   can vary from 2 to a million-fold, and can be obtained by measuring the potency (IC50) of a drug lead toward the drug target, versus the drug's potency (IC50) values against other pertinent targets (target pertinence is determined by the requirements of the biological system under investigation). A drug lead is deemed optimal when the parameters of potency, selectivity and cellular action are optimized with respect to each other.

25   In another embodiment, known drug leads from Steps 1-4 are found to be specific for targets that were not known to the researcher in step 2. This is also termed target discovery and validation, and occurs when steps 1-4 are repeated in an iterative fashion of any or all steps and the drug target is identified by the profiling array to, in fact, exist in an alternative signal transduction pathway, or to be a novel protein or enzyme in the pathway

30   originally under investigation. Thus, MPP arrays can identify the site of action of a drug lead, and can determine the relative selectivity of a drug for one drug target of a drug target pathway.

Step 6.   **Animal Model Assays**

In this embodiment, target cells (*e.g.*, tissue(s)) are removed from an animal model of the disease being targeted for treatment and lysed for testing. The lysate is contacted with nucleic acid sensor molecules either in a solid phase assay, a solution phase assay, or in a pathway profiling biosensor array format to assess the *in vivo* biological activity of a candidate drug identified by any of the previous steps or by some other method, on a target or pathway. Thus, in this embodiment, the physiological effect of a drug on a diseased or normal tissue is correlated with the *in vivo* mechanism of action of the drug.

Step 7.   Optimization of the Drug Lead

In one embodiment, drug-lead potency, specificity, and/or *in vivo* activity are optimized by an iterative repetition of any or all of steps 1-6. In one embodiment, steps 1-5 are repeated until the desired potency, selectivity and *in vivo* mechanism of action of a candidate drug are obtained.

Step 8.   Pharmacokinetic Studies

In one embodiment, the nucleic acid sensor molecules are used in pharmaco-kinetic studies, where the effect of a drug on the physiology of a cell, group of cells, tissue(s), organ(s), or animal model is assessed by obtaining blood, plasma, tissue, or a cell, and contacting this material with nucleic acid sensor molecules either in a solid phase (*e.g.*, ELISA), solution or array format to assess the *in vivo* pharmacological or toxicological activity of a compound. In this embodiment, the nucleic acid sensor molecules used are developed against the candidate drug itself, its metabolic products, and/or the metabolic products of proteins and small ligands involved in a xenobiotic or toxicological response to drug treatment.

In one embodiment, nucleic acid sensor molecules are employed to follow the fate of a drug or its metabolic by-products. In this embodiment, nucleic acid sensor molecules are generated to the drug and its metabolites. The drug is administered to the test animal either subcutaneously, intraperitoneally or by gavage. Subsequent to administration, the blood plasma or disease tissue is removed and its contents are screened for the remaining drug by Liquid chromatography (LC) or LC-mass spectrometry. Drug exposure is then determined as a function of time, dose and method of administration and is reported in values of half-life, bioavailability, AUC and Cmax. Metabolic products of a drug lead can be similarly followed.

Nucleic acid sensor molecules generated against enzymes or proteins known to those skilled in the art to be involved in drug metabolism (P450 enzymes, multi-drug transporter) can be used to follow the effect of a drug on xenobiotic or toxicological response to drug treatment.

5

Step 9. Optimization of the Drug Lead

In one embodiment, drug-lead potency, specificity, and/or *in vivo* activity, and pharmacokinetic, or toxological properties are optimized by an iterative repetition repetition of any or all of steps 1-7. In one embodiment, steps 1-7 are repeated until the

10 desired potency, selectivity and *in vivo* activity and pharmaco-kinetic, or toxicological properties of a candidate drug are obtained.

Step 10. Clinical Trials

In one embodiment, nucleic acid sensor molecules are used in clinical trials to

15 determine the fate of a drug in human or animal models, or used to follow the effect of drug treatment on a target or molecular pathway of choice, as described above. In one embodiment, the nucleic acid sensor molecules, in a solid phase assay (*e.g.*, ELISA format), a solution phase assay, or in a pathway profiling biosensor array format, are used to assess the *in vivo* biological activity of a drug being tested using lysed cell samples as described

20 above.

In another embodiment, the appropriate profiling biosensor is used *in vivo*, to monitor the effects of the compound on the patient, for example, by providing the biosensor in communication with a fiber optic probe inserted into the patient, or *ex vivo*, monitoring optical signals in a cell using a microscope based detection system. In another embodiment,

25 an *in vivo* assay is done by introducing a nucleic acid sensor molecule which retains its catalytic activity into a physiological system (*e.g.*, by injection at a target site in the body, through liposome carriers, and other means of administration routinely used in the art), obtaining cells from the physiological system and detecting the effect of the compound on the catalytic activity of the nucleic acid sensor molecule (*e.g.*, by evaluating the sequence of

30 the nucleic acid sensor molecule) as a means of determining the level, structure, or activity of a drug target, and relating the level, structure, or activity or the target molecules to the efficacy of the drug.

Step 11.   Optimization of the Drug Lead

In one embodiment, any or all of steps 1-10 are repeated to further optimize the properties of the candidate drug.

Step 12.   Diagnostic Applications

In one embodiment, individuals who would be suitable for treatment with the candidate drugs identified steps 1-11, are identified using nucleic acid sensor molecules in the diagnostic assays discussed previously.

Step 13.   Chemical Genomics

In one embodiment, nucleic acid sensor molecules are used in chemical genomic assays in which a drug or plurality of drug leads, with known or unknown physiological effects, and with unknown targets, are contacted with a physiological system and the site of action of the drug or plurality of drugs is determined using a plurality of the profiling biosensors described previously. Drug optimization then occurs as in steps 1-11.

5.   Use of a Profiling Biosensor in Target Molecule Separation

In addition to, or instead of, their use in detection methods, and drug discovery methods, the nucleic acid sensor molecules according to the invention can also be used to retrieve the target molecules which they specifically recognize. Additional embodiments exploiting the recognition capacity of the biosensors disclosed are contemplated and encompassed within the scope.

6.   Reagents for Generating and Using Nucleic Acid Sensor Molecules

In one embodiment, reagents are provided for generating and using nucleic acid sensor molecules. In one embodiment, a kit is provided comprising standardized reagents for making and/or using the nucleic acid sensor molecules according to the invention. In one embodiment, the kit comprises at least a first optical nucleic acid sensor molecule whose optical properties change upon recognition of a target molecule. In another embodiment, the kit additionally comprises any of: a control target molecule, a second nucleic acid sensor molecule which recognizess a different target molecule, suitable buffers, printed instructions, and combinations thereof. In a further embodiment, a nucleic acid sensor

molecule is provided with reagents for attaching a label and/or quencher or with reagents for attaching charge transfer molecules to the nucleic acid sensor molecule, which can sensitize the optical properties of the nucleic acid molecule to the presence of a target molecule.

5   In another embodiment, a composition is provided comprising a target molecule and a nucleic acid sensor molecule. The composition provides a reference against which to compare modified nucleic acid sensor molecules which recognizes to the same target, in order to select those with preferred cataytic activity or conformational change in the presence of the target. In a further embodiment, sets of complexes are provided. In still a

10   further embodiment, a set of pathway target molecules and nucleic acid sensor molecules are provided. In another embodiment, a set of profiling target molecules and nucleic acid sensor molecules are provided. In still a further embodiment, solid supports are provided for isolation of target molecules from nucleic acid sensor molecules.

      In yet another embodiment, a computer program product is provided comprising

15   stored data relating to optical signals generated by profiling and or pathway target molecules. In another embodiment, a means to compare this data to other optical signals is provided. In a further embodiment, the memory comprises data relating to patient information or chemical structure information relating to either target molecules or nucleic acid sensor molecules.

20   The nucleic acid sensor molecules and biosensors according to the invention are amenable for use with high throughput screening systems and methods and the use of the nucleic acid sensor molecules and biosensors in these systems and methods is encompassed within the scope. In one embodiment, the system is a robotic workstation, comprising, at least one of an: arrayer, microplate or microarray feeders, stackers, washers, and dispensers,

25   an optical system, a carousel, a conveyer for conveying microplates or microarrays from one part of the system to another (in a vertical or horizontal direction), a shaker system or other mixing system, a temperature control system, a synthesizer, a solid phase extraction system, and sample concentrators. Components of the robotic workstation can be part of a single integrated system or can be provided separately for use at any stage of the drug

30   optimization process according to the invention. In a further embodiment, the system comprises a processor connectable to the network which comprises or can access applications comprising stored data relating to profiling information obtained using nucleic acid sensor molecules according to the invention, and/or statistical applications, applications for performing structure/activity analysis of target molecules and nucleic acid sensor

molecules, applications for performing nucleic acid sequence alignment and simultaneous structure superposition of proteins (*e.g.*, MOE-Align'TM), applications for predicting binding conformations of molecules to receptor structures, and applications for controlling the processing functions of the robotic workstations.

5   The invention is further illustrated in the following non-limiting examples.

EXAMPLES

Example 1: General Procedures

A. Generating nucleic acid sensor molecules from pools of ribozymes
10   **comprised of randomized linker and target modulation domains.**

Direct selection of nucleic acid sensor molecules. Target modulated nucleic acid sensor molecules are isolated by *in vitro* selection. Pools of partially randomized ribozymes with 10^{15}-10^{17} unique sequences serve as the starting point for *in vitro* selection. As with the engineering approach, both the L1 ligase and hammerhead ribozyme are used as
15   platforms for the selection of allosterically-controlled molecules. Selections are designed to yield ribozymes that specifically respond to any target. Nucleic acid sensor molecules with cross-specificity (*i.e.* modulated by alternate target molecules, or by alternate ligand-bound states, or by alternate post-translationally modified forms of protein or peptides) are selected against by including the undesired form of the target in an initial negative selection step.
20   The specific sequence of operations comprising the selection experiment is outlined in Figure 1A and 1B for ligase and hammerhead-based selections, respectively

1.   **Pool preparation.** The starting library of DNA sequences is generated by automated chemical synthesis on a DNA synthesizer. This library of sequences is transcribed *in vitro* into RNA using T7 RNA polymerase, purified, and
25   captured onto beads using an oligonucleotide tag complementary to the 3'-end.

2.   **Negative selection incubation.** In the absence of the desired modulator (target), the RNA library are incubated together with the undesired form of the modulator and an arbitrary sequence oligonucleotide substrate (substrate 1). (During this incubation, non-modulated ribozymes undergo ligation (Figure 1A) or
30   cleavage (Figure 1B).

3.   **Positive selection incubation.** The undesired form of the modulator and oligonucleotide substrate 1 are removed by washing. The immobilized RNA

pool is then incubated under identical conditions but now in the presence of the intended modulator and a second biotinylated oligonucleotide substrate (substrate 2), in the case of ligase selections (Figure 1A).

4. **Partitioning on the basis of activity (coupled to amplification).**
5   Ligases active only in the presence of the desired modulator are isolated using streptavidin capture and selective PCR amplification (relying on sequence differences between substrates 1 and 2 to distinguish allosteric and non-allosteric activities). Hammerheads active only in presence of the desired modulator are isolated by gel electrophoresis.

10   5. **Purification.** PCR amplified DNA are purified and transcribed to yield an enriched pool for subsequent reselection.

        6. **Iteratively repeat.** Rounds of selection and amplification (steps 2-5) are repeated until functional members sufficiently dominate the resultant library.

15 Once NASMs have been selected as described above, they are characterized as follows:
        Nucleic acid sensor molecules which are derived from *in vitro* selection are tested as target modulated biosensors. The pool of NASMs is cloned into various plasmids that contain a T7 promoter transformed into *E. coli*. Individual NASM encoded DNA clones are isolated, linearized and the NASM is transcribed *in vitro* to generate NASM RNA. The
20 NASM RNAs are then tested in target modulation assays which determine the rate or extent of ribozyme modulation. For hammerhead NASMs, the extent of target dependent and independent reaction is determined by quantifying the extent of endonucleolytic cleavage of an oligonucleotide substrate. The extent of reaction can be followed by electrophoresing the reaction products on a denaturing PAGE gel, and subsequently analyzed by standard
25 radiometric methods. For Ligase NASMs, the extent of target dependent and independent reaction is determined by quantifying the extent of ligation of an oligonucleotide substrate, resulting in an increase in NASM molecular weight, as determined in denaturing PAGE gel electrophoresis.

        Individual NASM clones which display high target dependent switch factor values,
30 or high k_{act} rate values are subsequently chosen for further modification and evaluation.

        Hammerhead derived NASM clones are then further modified to render (the NASM sequences) them suitable for the optical detection applications that are described in detail below. In brief, these NASMs are used as fluorescent biosensors affixed to solid supports,

as fluorescent biosensors in homogeneous FRET-based assays, and as biosensors in SPR applications.

Ligase derived NASM clones are further modified to render them suitable for a number of detection platforms and applications; including, but not limited to, the PCR and
5   nucleotide amplification detection methods; fluorescent-based biosensors detectable in solution and chip formats; and as *in vivo*, intracellular detection biosensors.
The various detection applications of hammerhead ligase and intron-based NASMs are described in detail below.

10   **B. Preparation of an array of immobilized effector oligonucleotides or NASMs**

The following protocol describes a method for preparing an array of immobilized effector oligonucleotides with terminal amine groups attached to a solid substrate derivatized with aldehyde groups. The resulting array can then be used to spatially address (*i.e.*, the sequence of nucleotides for each effector oligonucleotide can be synthesized as a
15   cognate to the effector oligonucleotide binding domain of a nucleic acid sensor molecule specific for a particular target molecule) and immobilize the nucleic acid sensor molecules prior to their use in a solid-phase assay (see, *e.g.*, Zammatteo et al., 2000):

Protocol for attachment of effector oligonucleotides to aldehyde derivatized substrate
20   (www.arrayit.com):

1. Print discrete spots of solution containing effector oligonucleotides with amine-reactive terminal groups or linkers with terminal amine groups using microarraying pins, pipette, etc.

2. Allow spotted substrate to dry for 12 hrs. at room temperature and < 30% relative
25   humidity.

3. Rinse substrate 2 times in dH_2O with 0.2% SDS for 2 min. with vigorous agitation at room temperature.

4. Rinse substrate 1 time in dH_2O for 2 min. with vigorous agitation at room temperature.

30   5. Transfer substrate to boiling (100° C) dH_2O for 3 min. to denature DNA.

6. Dry substrate by centrifugation at 500x g for 1 min.

7. Treat substrate in 0.1 M $NaBH_4$ in phosphate buffered saline (PBS, pH 7) for 5 min. with mild agitation at room temperature.

8. Rinse substrate 2 times in dH_2O with 0.2% SDS for 1 min. with vigorous agitation at room temperature.

9. Rinse substrate 1 time in dH_2O for 2 min. with vigorous agitation at room temperature.

5   10. Transfer substrate to boiling (100 degrees C) dH_2O for 10 sec. to denature DNA.

11. Dry substrate by centrifugation at 500x g for 1 min.

12. Store effector oligonucleotide-bound substrate at 4 °C prior to hybridization.

The nucleic acid sensor molecules can be, e.g., those which possess either ligating or cleaving activity in the presence of a target molecule. (See, e.g., Figures 2A and B for the

10   ligater, Figure 5 for the cleaver).

In the case where it is desirable to immobilize an array of NASMs by direct attachment to a solid surface, the nucleic acid sensor molecules are bound to a solid substrate directly via their 3' termini. The attachment is accomplished by oxidation (using, e.g., Na periodate) of the 3' vicinal diol of the nucleic acid sensor molecule to an aldehyde

15   group. This aldehyde group will react with a hydrazide group to form a hydrazone bond. The hydrazone bond is quite stable to hydrolysis, etc., but can be further reduced (for example, by treatment with $NaBH_4$ or $NaCNBH_3$). The use of adipic acid dihydrazide (ADH, a bifunctional linker) to derivatize an aldehyde surface results in a hydrazide-derivatized surface which provides a linker of approximately 10 atoms between the

20   substrate surface and point of biomolecular attachment (see Ruhn et al., 1994; O'Shaughnessy, 1990; Roberston et al., 1972, Schluep et al., 1999; Chan et al., 1998). Preparation of a hydrazide-terminated surface via ADH treatment can be accomplished by treating an aldehyde-derivatized substrate according to the following protocol:

25   Protocol for ADH treatment of aldehyde substrate:

1. To 50 mL of 0.1 M phosphate buffer (pH 5) add 100-fold excess of adipic acid dihydrazide (ADH) relative to concentration of aldehyde groups on substrate surface.

2. Place substrate in a 50 mL tube containing the ADH in phosphate buffer and shake

30   mixture for 2 hrs.

3. Remove the substrate and wash 4 times with 0.1 M phosphate buffer (pH 7).

4. Reduce free aldehyde groups on substrate surface by placing substrate in a 50 mL tube containing a 25-fold excess of $NaBH_4$ or $NaCNBH_3$ in 0.1 M phosphate buffer.

5. Shake the mixture for 90 min.

6.   Wash 4 times with 0.1 M phosphate buffer (pH 7).

7.   Store ADH-treated substrates in 0.1 M phosphate buffer (pH 7) at 4° C.

Preparation of the nucleic acid molecules for specific coupling to the ADH-terminated

surface via their 3' termini can be accomplished according to the following protocol (see,

5   Proudnikov et al., 1996; Wu et al., 1996):

Protocol for Periodate oxidation of RNA:

1.   Dissolve up to 20 micrograms of RNA in 5 microliters of H20 at 20° C.

2.   Add 1 ML of 0.1 M NaIO$_4$ (~20-fold excess relative to RNA).

3.   Incubate for 30 min. in a light-tight tube or enclosure.

10   4.   Add 1 ML of 0.2 M Na sulphite (~2-fold excess relative to NaIO$_4$) to stop reaction.

5.   Incubate for 30 min. at room temperature.

6.   Ethanol precipitate or use spin-separation column to recover oxidized RNA.

Example 2.   Selection for a nucleic acid sensor molecule selective for the estrogen
15   **receptor LBD**

A nucleic acid sensor molecule which is modulated by the estrogen receptor (ER)

ligand binding domain (LBD) is obtained by *in vitro* selection methods to identify candidate

nucleic acid sensor molecules that are modulated by an estrogen receptor LBD.

20   The full length gene for the estrogen receptor is known. One source of the full-

length estrogen receptor clone is Acc. No. M12674 (see also Greene et al., Science

231:1150-54, 1986). The clone includes a 2092 nucleotide mRNA with the sequence

presented in Table 3 below:

Table 3

```
1     gaattccaaa  attgtgatgt  ttcttgtatt  tttgatgaag  gagaaatact  gtaatgatca
61    ctgtttacac  tatgtacact  ttaggccagc  cctttgtagc  gttatacaaa  ctgaaagcac
121   accggacccg  caggctcccg  gggcagggcc  ggggccagag  ctcgcgtgtc  ggcgggacat
181   gcgctgcgtc  gcctctaacc  tcgggctgtg  ctcttttcc   aggtggcccg  ccggtttctg
241   agccttctgc  cctgcgggga  cacggtctgc  accctgcccg  cggccacgga  ccatgaccat
301   gaccctccac  accaaagcat  ctgggatggc  cctactgcat  cagatccaag  ggaacgagct
361   ggagcccctg  aaccgtccgc  agctcaagat  ccccctggag  cggcccctgg  gcgaggtgta
421   cctggacagc  agcaagcccg  ccgtgtacaa  ctaccccgag  ggcgccgcct  acgagttcaa
481   cgccgcggcc  gccgccaacg  cgcaggtcta  cggtcagacc  ggcctcccct  acggccccgg
541   gtctgaggct  gcggcgttcg  gctccaacgg  cctggggggt  ttccccccac  tcaacagcgt
601   gtctccgagc  ccgctgatgc  tactgcaccc  gccgccgcag  ctgtcgcctt  tcctgcagcc
661   ccacggccag  caggtgccct  actacctgga  gaacgagccc  agcggctaca  cggtgcgcga
721   ggccggcccg  ccggcattct  acaggccaaa  ttcagataat  cgacgccagg  gtggcagaga
781   aagattggcc  agtaccaatg  acaagggaag  tatggctatg  gaatctgcca  aggagactcg
841   ctactgtgca  gtgtgcaatg  actatgcttc  aggctaccat  tatggagtct  ggtcctgtga
901   gggctgcaag  gccttcttca  agagaagtat  tcaaggacat  aacgactata  tgtgtccagc
961   caccaaccag  tgcaccattg  ataaaaacag  gaggaagagc  tgccaggcct  gccggctccg
1021  caaatgctac  gaagtgggaa  tgatgaaagg  tgggatacga  aaagaccgaa  gaggagggag
1081  aatgttgaaa  cacaagcgcc  agagagatga  tggggagggc  aggggtgaag  tggggtctgc
1141  tggagacatg  agagctgcca  acctttggcc  aagcccgctc  atgatcaaac  gctctaagaa
```

```
1201 gaacagcctg gccttgtccc tgacggccga ccagatggtc agtgccttgt tggatgctga
1261 gcccccata ctctattccg agtatgatcc taccagaccc ttcagtgaag cttcgatgat
1321 gggcttactg accaacctgg cagacaggga gctggttcac atgatcaact gggcgaagag
1381 ggtgccaggc tttgtggatt tgaccctcca tgatcaggtc caccttctag aatgtgcctg
1441 gctagagatc ctgatgattg gtctcgtctg gcgctccatg gagcacccag tgaagctact
1501 gtttgctcct aacttgctct tggacaggaa ccagggaaaa tgtgtagagg gcatggtgga
1561 gatcttcgac atgctgctgg ctacatcatc tcggttccgc atgatgaatc tgcagggaga
1621 ggagtttgtg tgcctcaaat ctattatttt gcttaattct ggagtgtaca catttctgtc
1681 cagcaccctg aagtctctgg aagagaagga ccatatccac cgagtcctgg acaagatcac
1741 agacactttg atccacctga tggccaaggc aggcctgacc ctgcagcagc agcaccagcg
1801 gctggcccag ctcctcctca tcctctccca catcaggcac atgagtaaca aaggcatgga
1861 gcatctgtac agcatgaagt gcaagaacgt ggtgcccctc tatgacctgc tgctggagat
1921 gctggacgcc caccgcctac atgcgcccac tagccgtgga ggggcatccg tggaggagac
1981 ggaccaaagc cacttggcca ctgcgggctc tacttcatcg cattccttgc aaaagtatta
2041 catcacgggg gaggcagagg gtttccctgc cacagtctga gagctccctg gc (SEQ ID
NO:9)
```

The polynucleotide encodes a polypeptide with the amino acid sequence presented in Table 4 below:

Table 4

```
MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYG
QTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAGPPA
FYRPNSDNRRQGGRERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCP
ATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPL
MIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDL
TLHDQVHLLECAWLEILMIGLVWRSMEHPVKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEE
FVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMS
NKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSHSLQKYYITGEAEGFP
ATV (SEQ ID NO:10)
```

The gene encoding either full length ER or the ligand binding domain is cloned and expressed in BL21 (DE3)-pLysS *E. coli* cells [Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. Cell. 1998 Dec 23;95(7):927-37]. Human ER alpha LBD (residues 297-554) are purified from estradiol-sepharose column by published procedures [Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. Cell. 1998 Dec 23;95(7):927-37].

Selection of nucleic acid sensor molecules which are activated by ER-LBD not bound to ligand: A library of up to 10^{17} variants of *in vitro* synthesized (1 μM) nucleic acid sensor molecules is allowed to react with purified apo-ER-LBD at a final concentration of 1uM. Selection of catalytic nucleic acid sensor molecules and optionally, generation of an optical NASM, is carried out by procedures outlined in prior examples and elsewhere herein.

Selection of nucleic acid sensor molecules which are modulated by the ER-LBD-Estradiol complex:

Stable complexes of ER-LBD and estradiol ligand are formed with from 1-10 equivalents of ligand. A library of up to 10^{17} variants of *in vitro* synthesized ribozymes is then allowed to react with purified ER-LBD-Estradiol at a final complex concentration of 1uM. Selection of allosterically activated ribozymes is carried out by procedures outlined

5   the detailed description. Selection of nucleic acid sensor molecules which are modulated by the ER-LBD-Tamoxifen complex stable complexes of ER-LBD and tamoxifen ligand are formed with from 1-10 equivalents of ligand. A library of up to 10^{17} variants of *in vitro* synthesized ribozymes (1 µM containing a plurality of potential target modulation domains and linker domains coupled to the catalytic domain of the ribozyme, is then allowed to react

10   with purified ER-LBD-Tamoxifen at a final complex concentration of 1uM. Selection of catalytic nucleic acid sensor molecules target modulated ribozymes (nucleic acid sensor molecules) is carried out by procedures outlined in prior examples.

Example 3. Selection for a library of nucleic acid sensor molecules which signal the
15   **presence of all known nuclear hormone receptor LBDs:**

A wide variety of nuclear hormone receptor ("NHR") ligand binding domains and their ligands, many of which are described in Table 5 below, are known for which a nucleic acid sensor molecule can be selected.

20   **Table 5**

Symbol	Description	Ligand
AIB3	nuclear receptor coactivator RAP250; peroxisome proliferator-activated receptor interacting protein; thyroid hormone receptor binding protein	thyroid hormone
AR	androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)	dihydroxytestosteron
C1D	nuclear DNA-binding protein	
ESR1	estrogen receptor 1	estrogen
ESR2	estrogen receptor 2 (ER beta)	estrogen
ESRRA	estrogen-related receptor alpha	estrogen and TFIIB
ESRRB	estrogen-related receptor beta	estrogen and TFIIB
ESRRG	estrogen-related receptor gamma	estrogen and TFIIB
HNF4A	hepatocyte nuclear factor 4, alpha	
HNF4G	hepatocyte nuclear factor 4, gamma similar to retinoid X receptor, alpha (H. sapiens) similar to nuclear receptor subfamily 1, group D, member 1 (H. sapiens)	

NCOA1	nuclear receptor coactivator 1	Binds to steroid hormone receptors
NCOR1	nuclear receptor co-repressor 1	Thyroid hormone receptor without TH
NCOR2	nuclear receptor co-repressor 2	RXR without retinoic acid and THR without TH
NR0B1	nuclear receptor subfamily 0, group B, member 1	
NR0B2	nuclear receptor subfamily 0, group B, member 2	
NR1D1	nuclear receptor subfamily 1, group D, member 1	
NR1H2	nuclear receptor subfamily 1, group H, member 2	
NR1H3	nuclear receptor subfamily 1, group H, member 3	Interacts with RXR
NR1H4	nuclear receptor subfamily 1, group H, member 4	Bile acid, farnesol, or chenodoxycholic acid
NR1I2	nuclear receptor subfamily 1, group I, member 2	pregnane
NR1I3	nuclear receptor subfamily 1, group I, member 3	androstane S
NR2C1	nuclear receptor subfamily 2, group C, member 1	
NR2C2	nuclear receptor subfamily 2, group C, member 2	
NR2E1	nuclear receptor subfamily 2, group E, member 1	
NR2E3	nuclear receptor subfamily 2, group E, member 3	
NR2F1	nuclear receptor subfamily 2, group F, member 1	
NR2F2	nuclear receptor subfamily 2, group F, member 2	
NR2F6	nuclear receptor subfamily 2, group F, member 6	Thyroid hormone
NR3C1	nuclear receptor subfamily 3, group C, member 1	glutocorticoid receptor, cortisol, corticosterone
NR3C2	nuclear receptor subfamily 3, group C, member 2	Aldosterone
NR4A1	nuclear receptor subfamily 4, group A, member 1	
NR4A2	nuclear receptor subfamily 4, group A, member 2	
NR4A3	nuclear receptor subfamily 4, group A, member 3	
NR5A1	nuclear receptor subfamily 5, group A, member 1	
NR5A2	nuclear receptor subfamily 5, group A, member 2	
NR6A1	nuclear receptor subfamily 6, group A, member 1	
PAX8	paired box gene 8	
PGR	progesterone receptor	progesterone
PPARA	peroxisome proliferative activated receptor, alpha	nafenopin, clofibrate, WY14643
PPARBP	PPAR binding protein	binds to PPAR gamma
PPARD	peroxisome proliferative activated receptor, delta	WY1463
PPARG	peroxisome proliferative activated receptor, gamma	9-HODE, 13-HODE
PTHR1	parathyroid hormone receptor 1	parathyroid hormone
RARA	retinoic acid receptor, alpha	retinoic acid
RARB	retinoic acid receptor, beta	retinoic acid
RARG	retinoic acid receptor, gamma	retinoic acid
RORA	RAR-related orphan receptor A	

RORB	RAR-related orphan receptor B	
RORC	RAR-related orphan receptor C	
RXRA	retinoid X receptor, alpha	9-cis retonoic acid, complexes with activated VDR and THR
RXRB	retinoid X receptor, beta	9-cis retonoic acid, complexes with activated VDR and THR
RXRG	retinoid X receptor, gamma	9-cis retonoic acid, complexes with activated VDR and THR
SMAP	thyroid hormone receptor coactivating protein	activated THR
THRA	thyroid hormone receptor, alpha (avian erythroblastic leukemia viral (v-erb-a) oncogene homolog)	thyroid hormone
THRB	thyroid hormone receptor, beta (avian erythroblastic leukemia viral (v-erb-a) oncogene homolog 2)	thyroid hormone
TNRC11	trinucleotide repeat containing 11 (THR-associated protein, 230 kDa subunit)	activated THR
TRAP150	thyroid hormone receptor-associated protein, 150 kDa subunit	activated THR
TRAP240	thyroid hormone receptor-associated protein, 240 kDa subunit	activated THR
TRAP95	thyroid hormone receptor-associated protein, 95-kD subunit	activated THR
TRIP13	thyroid hormone receptor interactor 13	inactive THR
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	vitamin D

N-terminally GST-tagged or N-/C-terminally His-tagged nuclear hormone receptor ligand binding domains, defined on the basis of structural homology are cloned and expressed in BL21 (DE3)-pLysS *E. coli* cells, or are cloned and expressed in standard baculovirus expression systems.

Human NHR LBDs (homologous to ER-alpha residues including the region aa 297-554) are purified from GSH-sepharose or nickel affinity columns by published procedures available from the manufacturers. LBDs are produced in a either a parallel or serial fashion and the purified proteins are stored in buffer containing 50 mM TrisHCl, 1 mM EDTA, 1mM DTT and 50-250 NaCl/SCN salt, pH 7 to pH 8.5, 10% glycerol or other stabilizing agent. Protein sequence and MW is verified by electrospray LC-MS mass spectrometry.

1. Selection of nucleic acid sensor molecules which are modulated by NHR-LBD not bound to ligand: A library of up to 10^{17} variants of *in vitro* synthesized ribozymes (1 μM) containing a plurality of potential target modulation domains and linker domains

coupled to the catalytic domain of the ribozyme, is allowed to react with purified apo-NHR-LBDs at a final concentration of 1 uM LBD. Selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in the Detailed Description. Selections are carried out in parallel fashion. Selections can also be carried out in mixed pools of

5 anywhere from 5-10 NHR-LBDs. In the final rounds of nucleic acid sensor molecule selection, the RNA pools may separated into aliquots which may then be used to carry out *in vitro* selection against single NHR-LBD proteins to yield unique nucleic acid sensor molecules selective for multiple NHR-LBDs.

2. Selection of nucleic acid sensor molecules which are activated by ligand bound

10 forms of NHR-LBDs.

Stable complexes of each NHR-LBD are formed with from 1-10 equivalents of ligand. A library of up to 10^{17} variants of *in vitro* synthesized ribozymes (1 μM) containing a plurality of potential target modulation domains and linker domains coupled to the catalytic domain of the ribozyme is then allowed to react with purified NHR-LBD-Ligand

15 complexes at a final complex concentration of 1uM. Selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in the Detailed Description and Example 1 below. Selections are carried out in parallel fashion. Selections can also be carried out in mixed pools of anywhere from 5-10 NHR-LBD-Ligand complexes. In the final rounds of nucleic acid sensor molecule selection, the RNA pools may separated into

20 aliquots which may then be used to carry out *in vitro* selection against single NHR-LBD-Ligand complexes to yield unique nucleic acid sensor molecules selective for all NHR-LBDs, bound to their ligands.

Thus, the invention provides an *in vitro* selection protocol against purified LBDs, bound to their ligands or not, for each known NHR. *In vitro* selections can be carried out

25 with less than 1 mg of the purified forms of the LBDs. In addition the selection of nucleic acid sensor molecules can be done *in vitro* with mixed pools of LBD and subsequently deconvoluted after selection is complete. Alternatively, the final selection can be carried out with fractionally purified extracts containing a slight excess of recombinant LBD. In one embodiment the LBD is expressed in *E. coli*, or insect cell lines or mammalian cell

30 lines. In another embodiment, the selection is carried out in cell free lysates in which the LBD is expressed in an *in vitro* transcription-translation procedure such as is described in the literature or can be purchased using common reagents from Roche or Promega. In another embodiment, the fractionated or purified LBDs are combined with known ligands, (as described above) agonist, antagonists or partial agonist/antagonists to form stable

complexes, and these complexes are then used for *in vitro* selection of nucleic acid sensor molecules. Upon interaction of the nucleic acid sensor molecule with the NHR-LBD, a signal will be generated detectable to an external monitoring device. In this manner, the activation state of any or all NHRs can be monitored *in vivo* or *in vitro* as will be described in detail in subsequent examples.

Example 4. Selection for a nucleic acid sensor molecule selective for the Beta-2 adrenergic receptor.

The full-length gene for the Beta-2 adrenergic receptor is described (Emorine et al., Proc. Natl. Acad. Sci. USA 84:6995-99, 1987) and is available at Acc. No. AAA88017. The nucleic acid sequence is set forth in Table 6 below:

Table 6

```
GCACCGCGAGCCCCTAGCACCCGACAAGCTGAGTGTGCAGGACGAGTCCCCACCACACCC
ACACCACAGCCGCTGAATGAGGCTTCCAGGCGTCCGCTCGCGGCCCGCAGAGCCCCGCCG
TGGGTCCGCCCGCTGAGGCGCCCCCAGCCAGTGCGCTTACCTGCCAGACTGCGCGCCATG
GGGCAACCCGGGAACGGCAGCGCCTTCTTGCTGGCACCCAATGGAAGCCATGCGCCGGAC
CACGACGTCACGCAGGAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTCT
CTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCATTGCCAAGTTC
GAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCACTGGCCTGTGCTGATCTGGTC
ATGGGCCTGGCAGTGGTGCCCTTTGGGGCCGCCCATATTCTTATGAAAATGTGGACTTTT
GGCAACTTCTGGTGCGAGTTTTGGACTTCCATTGATGTGCTGTGCGTCACGGCCAGCATT
GAGACCCTGTGCGTGATCGCAGTGGATCGCTACTTTGCCATTACTTCACCTTTCAAGTAC
CAGAGCCTGCTGACCAAGAATAAGGCCCGGGTGATCATTCTGATGGTGTGGATTGTGTCA
GGCCTTACCTCCTTCTTGCCCATTCAGATGCACTGGTACCGGGCCACCCACCAGGAAGCC
ATCAACTGCTATGCCAATGAGACCTGCTGTGACTTCTTCACGAACCAAGCCTATGCCATT
GCCTCTTCCATCGTGTCCTTCTACGTTCCCCTGGTGATCATGGTCTTCGTCTACTCCAGG
GTCTTTCAGGAGGCCAAAAGGCAGCTCCAGAAGATTGACAAATCTGAGGGCCGCTTCCAT
GTCCAGAACCTTAGCCAGGTGGAGCAGGATGGGCGGACGGGGCATGGACTCCGCAGATCT
TCCAAGTTCTGCTTGAAGGAGCACAAAGCCCTCAAGACGTTAGGCATCATCATGGGCACT
TTCACCCTCTGCTGGCTGCCCTTCTTCATCGTTAACATTGTGCATGTGATCCAGGATAAC
CTCATCCGTAAGGAAGTTTACATCCTCCTAAATTGGATAGGCTATGTCAATTCTGGTTTC
AATCCCCTTATCTACTGCCGGAGCCCAGATTTCAGGATTGCCTTCCAGGAGCTTCTGTGC
CTGCGCAGGTCTTCTTTGAAGGCCTATGGGAATGGCTACTCCAGCAACGGCAACACAGGG
GAGCAGAGTGGATATCACGTGGAACAGGAGAAAGAAAATAAACTGCTGTGTGAAGACCTC
CCAGGCACGGAAGACTTTGTGGGCCATCAAGGTACTGTGCCTAGCGATAACATTGATTCA
CAAGGGAGGAATTGTAGTACAAATGACTCACTGCTGTAAAGCAGTTTTTCTACTTTTAAA
GACCCCCCCCCCCCAACAGAACACTAAACAGACTATTTAACTTGAGGGTAATAAACTTA
GAATAAAATTGTAAAAATTGTATAGAGATATGCAGAAGGAAGGGCATCCTTCTGCCTTTT
TTATTTTTTTAAGCTGTAAAAAGAGAGAAAACTTATTTGAGTGATTATTTGTTATTTGTA
CAGTTCAGTTCCTCTTTGCATGGAATTTGTAAGTTTATGTCTAAAGAGCTTTAGTCCTAG
AGGACCTGAGTCTGCTATATTTTCATGACTTTTCCATGTATCTACCTCACTATTCAAGTA
TTAGGGGTAATATATTGCTGGTAATTTGTATCTGAAGGAGATTTTCCTTCCTACACCCTT
GGACTTGAGGATTTTGAGTATCTCGGACCTTTCAGCTGTGAACATGGACTCTTCCCCCAC
TCCTCTTATTTGCTCACACGGGGTATTTTAGGCAGGGATTTGAGGAGCAGCTTCAGTTGT
TTTCCCGAGCAAAGGTCTAAAGTTTACAGTAAATAAAATGTTTGACCATG (SEQ ID NO:11)
```

The amino acid sequence of the polypeptide encoded by the nucleic acid sequence is set forth in Table 7 below:

Table 7

```
1    mgqpgngsaf llapngshap dhdvtqqrde vwvvgmgivm slivlaivfg nvlvitaiak
61   ferlqtvtny fitslacadl vmglavvpfg aahilmkmwt fgnfwcefwt sidvlcvtas
121  ietlcviavd ryfaitspfk yqslltknka rviilmvwiv sgltsflpiq mhwyrathqe
181  aincyanetc cdfftnqaya iassivsfyv plvimvfvys rvfqeakrql qkidksegrf
241  hvqnlsqveq dgrtghglrr sskfclkehk alktlgiimg tftlcwlpff ivnivhviqd
301  nlirkevyil lnwigyvnsg fnpliycrsp dfriafqell clrrsslkay gngyssngnt
361  geqsgyhveq ekenkllced lpgtedfvgh qgtvpsdnid sqgrncstnd sll (SEQ ID
NO:12)
```

The gene encoding either the full-length Beta-2 adrenergic receptor or cytoplasmic loop II or III or helix VII is cloned, expressed and purified from *E. coli* or baculovirus infected cells (Hampe, et al., J Biotechnol 77:219-234 (2000)) according to published procedures, and incorporated into detergent micelles to simulate the cellular milieu (Min, et al., J Biol Chem 268:9400-9404 (1993)). A library of up to 10^{17} variants of *in vitro* synthesized ribozymes (1 μM) containing a plurality of potential target modulation domains and linker domains coupled to the catalytic domain of the ribozyme is allowed to react with purified Beta-2 adrenergic receptor at a final concentration of 1 uM. Selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in Example 1.

Selection of nucleic acid sensor molecules which are activated by the butoxamine-Beta-2 adrenergic complex. 1:1 complexes of butoxamine and purified Beta-2 adrenergic receptor are formed and selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in Example 1.

Selection of nucleic acid sensor molecules which are activated by the isoproterenol-Beta-2 adrenergic complex is accomplished as follows. 1:1 complexes of isoproterenol and purified Beta-2 adrenergic receptor are formed and selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in Example 1 and the Detailed Description.

Example 5: Selection for a library of nucleic acid sensor molecules which signal the presence of all known GPCRs

The full-length gene sequences for over 400 G-protein coupled receptors ("GPCR") is known. GPCR polypeptides, peptides and peptide fragments are also know as set forth in Table 8 and Table 9 below. The entire gene, or peptides derived from these sequences, is N-terminally tagged or N-/C- terminally His-tagged and cloned, expressed, and purified, or synthesized by chemical means. All 400 plus GPCRs are produced in either a serial or parallel fashion and the purified proteins or peptides stored in buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 50-250 mM NaCl/SCN salt, pH 7 to pH 8.5, 10% glycerol or other stabilizing agent.

Table 8

	ref|NP_006134.1|	G protein-coupled receptor 19 [Homo sapiens]
	ref|XP_049562.1|	G protein-coupled receptor 19 [Homo sapiens]
5	ref|NP_004876.1|	neuropeptide G protein-coupled receptor; n...
	ref|XP_011102.1|	46228 [Homo sapiens] >gi|14723215|ref|XP_0...
	ref|NP_057624.1|	G protein-coupled receptor 72; reserved; G...
	ref|XP_011520.3|	orexin receptor 2 [Homo sapiens]
	ref|NP_001517.1|	orexin receptor 2 [Homo sapiens]
10	ref|NP_001471.1|	galanin receptor 1; Galanin receptor [Homo...
	ref|NP_071429.1|	neuropeptide FF 1; RFamide-related peptide...
	ref|XP_005747.4|	tachykinin receptor 2 [Homo sapiens]
	ref|NP_001048.1|	tachykinin receptor 2; Tachykinin receptor...
	ref|NP_000901.1|	neuropeptide Y receptor Y2 [Homo sapiens] ...
15	ref|NP_003848.1|	galanin receptor 2 [Homo sapiens] >gi|1365...
	ref|XP_004030.2|	adrenergic, beta-2-, receptor, surface [Ho...
	ref|NP_000015.1|	adrenergic, beta-2-, receptor, surface [Ho...
	ref|XP_001777.1|	orexin receptor 1 [Homo sapiens]
	ref|XP_011871.3|	neuropeptide FF 1; RFamide-related peptide...
20	ref|NP_001516.1|	orexin receptor 1 [Homo sapiens]
	ref|NP_001041.1|	somatostatin receptor 2 [Homo sapiens] >gi...
	ref|NP_001040.1|	somatostatin receptor 1 [Homo sapiens] >gi...
	ref|NP_001044.1|	somatostatin receptor 5 [Homo sapiens]
	ref|XP_012565.1|	somatostatin receptor 5 [Homo sapiens]
25	ref|NP_115940.1|	G protein-coupled receptor; G protein-coup...
	ref|XP_037563.1|	G protein-coupled receptor [Homo sapiens]
	ref|NP_001050.1|	tachykinin receptor 3; NK-3 receptor; neur...
	ref|XP_011942.1|	prolactin-releasing hormone receptor [Homo...
	ref|XP_017624.1|	G protein-coupled receptor 58 [Homo sapiens]
30	ref|NP_004239.1|	prolactin-releasing hormone receptor [Homo...
	ref|NP_071640.1|	histamine receptor H2; gastric receptor 1 ...
	ref|NP_055441.1|	G protein-coupled receptor 58 [Homo sapiens]
	ref|XP_009594.2|	somatostatin receptor 4 [Homo sapiens]
	ref|NP_003605.1|	galanin receptor 3; galanin receptor, fami...
35	ref|NP_001049.1|	tachykinin receptor 1, isoform long; Tachy...
	ref|XP_039747.1|	opioid receptor, mu 1 [Homo sapiens] >gi|1...
	ref|NP_000905.1|	opioid receptor, mu 1 [Homo sapiens]
	ref|XP_004341.2|	53355 [Homo sapiens]
	ref|XP_052174.1|	50635 [Homo sapiens]
40	ref|XP_052175.1|	5-hydroxytryptamine (serotonin) receptor 4...
	ref|XP_052165.1|	5-hydroxytryptamine (serotonin) receptor 4...
	ref|XP_052164.1|	50636 [Homo sapiens] >gi|14732317|ref|XP_0...
	ref|NP_000861.1|	5-hydroxytryptamine (serotonin) receptor 4...
	ref|NP_001043.1|	somatostatin receptor 4 [Homo sapiens]
45	ref|NP_000721.1|	cholecystokinin A receptor [Homo sapiens] ...
	ref|NP_006670.1|	putative opioid receptor, neuromedin K (ne...
	ref|NP_055442.1|	G protein-coupled receptor 57 [Homo sapiens]
	ref|NP_000698.1|	arginine vasopressin receptor 1B; arginine...
	ref|NP_001718.1|	bombesin-like receptor 3 [Homo sapiens] >g...
50	ref|XP_040306.1|	similar to SOMATOSTATIN RECEPTOR TYPE 2 (S...
	ref|NP_056542.1|	tachykinin receptor 1, isoform short; Tach...
	ref|NP_001042.1|	somatostatin receptor 3 [Homo sapiens] >gi...
	ref|NP_000722.1|	cholecystokinin B receptor [Homo sapiens]
	ref|NP_000789.1|	dopamine receptor D5; Dopamine receptor D1...
55	ref|NP_000612.1|	5-hydroxytryptamine (serotonin) receptor 2...
	ref|NP_004215.1|	G protein-coupled receptor 50 [Homo sapiens]
	ref|XP_010228.2|	G protein-coupled receptor 50 [Homo sapiens]
	ref|NP_000907.1|	oxytocin receptor [Homo sapiens]
	ref|XP_052179.1|	oxytocin receptor [Homo sapiens] >gi|14725...
60	ref|NP_005192.1|	chemokine (C-C motif) receptor 8; chemokin...
	ref|NP_000670.1|	adrenergic, alpha-1B-, receptor; adrenergi...

	ref|XP_046588.1|	G protein-coupled receptor slt [Homo sapie...
	ref|NP_000671.1|	adrenergic, alpha-1A-, receptor; adrenergi...
	ref|NP_110411.1|	brain expressed G-protein-coupled receptor...
	ref|XP_003199.2|	growth hormone secretagogue receptor [Homo...
5	ref|XP_017623.1|	G protein-coupled receptor 57 [Homo sapiens]
	ref|NP_005949.1|	melatonin receptor 1A; melatonin receptor ...
	ref|NP_115892.1|	G protein-coupled receptor slt; melanin-co...
	ref|NP_000903.1|	opioid receptor, kappa 1; Opiate receptor,...
	ref|NP_000900.1|	neuropeptide Y receptor Y1; Neuropeptide Y...
10	ref|XP_011716.2|	similar to opioid receptor, kappa 1; Opiat...
	ref|XP_011707.2|	adrenergic, alpha-1A-, receptor [Homo sapi...
	ref|XP_048085.1|	adrenergic, alpha-1A-, receptor [Homo sapi...
	ref|XP_048084.1|	adrenergic, alpha-1A-, receptor [Homo sapi...
	ref|NP_000785.1|	dopamine receptor D1 [Homo sapiens]
15	ref|XP_048082.1|	adrenergic, alpha-1A-, receptor [Homo sapi...
	ref|NP_003292.1|	thyrotropin-releasing hormone receptor [Ho...
	ref|NP_005305.1|	gastrin-releasing peptide receptor [Homo s...
	ref|XP_006335.4|	dopamine receptor D2 [Homo sapiens]
	ref|XP_006334.3|	dopamine receptor D2longer [Homo sapiens]
20	ref|NP_000786.1|	dopamine receptor D2 [Homo sapiens] >gi|14...
	ref|XP_036647.1|	dopamine receptor D2 [Homo sapiens] >gi|14...
	ref|XP_041422.1|	similar to dopamine receptor D2 (H. sapien...
	ref|NP_057658.1|	dopamine receptor D2longer [Homo sapiens] ...
	ref|NP_000697.1|	arginine vasopressin receptor 1A; V1a vaso...
25	ref|NP_002502.1|	neuromedin B receptor [Homo sapiens]
	ref|XP_018475.1|	neuromedin B receptor [Homo sapiens]
	ref|NP_062874.1|	5-hydroxytryptamine receptor 7, isoform b;...
	ref|NP_000730.1|	cholinergic receptor, muscarinic 2; muscar...
	ref|NP_062873.1|	5-hydroxytryptamine receptor 7, isoform d;...
30	ref|NP_000863.1|	5-hydroxytryptamine receptor 7, isoform a;...
	ref|NP_000667.1|	adenosine A2b receptor [Homo sapiens] >gi|...
	ref|NP_000675.1|	beta-1-adrenergic receptor [Homo sapiens]
	ref|NP_005963.1|	pancreatic polypeptide receptor 1 [Homo sa...
	ref|NP_000732.1|	cholinergic receptor, muscarinic 4; muscar...
35	ref|XP_039923.1|	44527 [Homo sapiens]
	ref|NP_000787.1|	dopamine receptor D3 [Homo sapiens]
	ref|XP_011027.3|	dopamine receptor D3 [Homo sapiens]
	ref|NP_061822.1|	G protein-coupled receptor 14 [Homo sapiens]
	ref|NP_000731.1|	cholinergic receptor, muscarinic 3; muscar...
40	ref|NP_000669.1|	adrenergic, alpha-1D-, receptor; adrenergi...
	ref|NP_005282.1|	G protein-coupled receptor 17 [Homo sapien...
	ref|XP_048332.1|	similar to purinergic receptor (family A g...
	ref|XP_041897.1|	similar to G protein-coupled receptor 17 (...
	ref|NP_005152.1|	angiotensin receptor-like 1 [Homo sapiens]...
45	ref|NP_000016.1|	adrenergic, beta-3-, receptor [Homo sapien...
	ref|NP_001286.1|	chemokine (C-C motif) receptor 1; macropha...
	ref|NP_000902.1|	opioid receptor, delta 1 [Homo sapiens]
	ref|NP_005758.1|	purinergic receptor (family A group 5) [Ho...
	ref|XP_006296.1|	cholinergic receptor, muscarinic 4 [Homo s...
50	ref|XP_006058.1|	similar to MUSCARINIC ACETYLCHOLINE RECEPT...
	ref|NP_000729.1|	cholinergic receptor, muscarinic 1; muscar...
	ref|NP_000904.1|	opiate receptor-like 1; opiod receptor-lik...
	ref|NP_006630.1|	cysteinyl leukotriene receptor 1 [Homo sap...
	ref|NP_001828.1|	chemokine (C-C motif) receptor 3 [Homo sap...
55	ref|NP_000530.1|	rhodopsin; rhodopsin (retinitis pigmentosa...
	ref|NP_009154.1|	putative G protein coupled receptor [Homo ...
	ref|XP_009882.2|	adenosine A2a receptor [Homo sapiens]
	ref|XP_045486.1|	adrenergic, alpha-1D-, receptor [Homo sapi...
	ref|NP_000397.1|	gonadotropin-releasing hormone receptor; g...
60	ref|NP_000666.2|	adenosine A2a receptor; adenosine A2 recep...
	ref|XP_002299.1|	G protein-coupled receptor 45 [Homo sapiens]

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        ref|NP_003958.1|  putative neurotransmitter receptor [Homo s...
        ref|NP_000854.1|  5-hydroxytryptamine (serotonin) receptor 1...
        ref|XP_001811.2|  opioid receptor, delta 1 [Homo sapiens] >g...
        ref|NP_005950.1|  melatonin receptor 1B; melatonin receptor ...
    5   ref|NP_001548.1|  interleukin 8 receptor, beta [Homo sapiens]
        ref|NP_064552.1|  neuromedin U receptor 2 [Homo sapiens]
        ref|NP_009158.1|  G protein-coupled receptor 45 [Homo sapiens]
        ref|NP_000625.1|  interleukin 8 receptor, alpha; chemokine (...
        ref|XP_017622.1|  putative neurotransmitter receptor [Homo s...
   10   ref|NP_036257.1|  cholinergic receptor, muscarinic 5; muscar...
        ref|NP_000743.1|  leukotriene b4 receptor (chemokine recepto...
        ref|NP_000788.1|  dopamine receptor D4 [Homo sapiens]
        ref|XP_006145.2|  dopamine receptor D4 [Homo sapiens]
        ref|XP_001543.1|  G protein-coupled receptor 52 [Homo sapiens]
   15   ref|XP_009663.1|  G protein-coupled receptor 8 [Homo sapiens]
        ref|XP_007212.1|  purinergic receptor (family A group 5) [Ho...
        ref|NP_006165.1|  neuropeptide Y receptor Y5 [Homo sapiens]
        ref|NP_002522.1|  neurotensin receptor 1 [Homo sapiens]
        ref|XP_003692.2|  5-hydroxytryptamine (serotonin) receptor 1...
   20   ref|XP_009612.2|  neurotensin receptor 1 [Homo sapiens]
        ref|NP_005277.1|  G protein-coupled receptor 8 [Homo sapiens]
        ref|NP_000515.1|  5-hydroxytryptamine (serotonin) receptor 1...
        ref|NP_001707.1|  Burkitt lymphoma receptor 1, isoform 1; C-...
        ref|NP_005675.1|  G protein-coupled receptor 52 [Homo sapiens]
   25   ref|NP_000852.1|  histamine receptor H1; histamine receptor,...
        ref|NP_000639.1|  chemokine (C-C motif) receptor 2; chemokin...
        ref|NP_116743.1|  Burkitt lymphoma receptor 1, isoform 2; C-...
        ref|NP_006574.1|  retinal pigment epithelium-derived rhodops...
        ref|NP_031395.1|  G-protein coupled receptor [Homo sapiens] ...
   30   ref|XP_009373.1|  formyl peptide receptor-like 2 [Homo sapie...
        ref|NP_076917.1|  5-hydroxytryptamine (serotonin) receptor 5...
        ref|XP_005280.2|  G protein-coupled receptor 7 [Homo sapiens]
        ref|XP_039818.1|  G protein-coupled receptor 91 [Homo sapien...
        ref|NP_006164.1|  neuropeptide Y receptor Y6 (pseudogene) [H...
   35   ref|XP_012748.2|  galanin receptor 1 [Homo sapiens] >gi|1476...
        ref|NP_000862.1|  5-hydroxytryptamine (serotonin) receptor 6...
        ref|NP_005281.1|  G protein-coupled receptor 15 [Homo sapien...
        ref|XP_010406.1|  angiotensin receptor 2 [Homo sapiens] >gi|...
        ref|NP_001328.1|  chemokine (C-X3-C) receptor 1; chemokine (...
   40   ref|NP_002554.1|  purinergic receptor P2Y, G-protein coupled...
        ref|NP_004942.1|  EBV-induced G protein-coupled receptor 2; ...
        ref|NP_000672.1|  adrenergic, alpha-2A-, receptor [Homo sapi...
        ref|XP_005827.3|  beta-1-adrenergic receptor [Homo sapiens]
        ref|NP_005288.1|  G protein-coupled receptor 24 [Homo sapien...
   45   ref|NP_002021.2|  formyl peptide receptor-like 2 [Homo sapie...
        ref|NP_005276.1|  G protein-coupled receptor 7 [Homo sapiens]
        ref|XP_010009.2|  similar to somatostatin receptor-like prot...
        ref|NP_003458.1|  chemokine (C-X-C motif), receptor 4 (fusin...
        ref|NP_000673.1|  adrenergic, alpha-2B-, receptor [Homo sapi...
   50   ref|XP_051229.1|  similar to C-X-C CHEMOKINE RECEPTOR TYPE 4...
        ref|NP_001453.1|  formyl peptide receptor-like 1; lipoxin A4...
        ref|NP_006047.1|  G protein-coupled receptor 66 [Homo sapien...
        ref|NP_000856.1|  5-hydroxytryptamine (serotonin) receptor 1...
        ref|XP_048737.1|  41064 [Homo sapiens]
   55   ref|NP_005499.1|  chemokine (C-C motif) receptor 4; chemokin...
        ref|NP_000638.1|  chemokine (C-C motif) receptor 2; chemokin...
        ref|XP_009664.1|  opiate receptor-like 1 [Homo sapiens] >gi|...
        ref|NP_005285.1|  G protein-coupled receptor 21 [Homo sapien...
        ref|XP_009561.2|  34426 [Homo sapiens]
   60   ref|NP_004063.1|  chemokine-like receptor 1 [Homo sapiens] >...
        ref|XP_035769.1|  chemokine-like receptor 1 [Homo sapiens]
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     ref|NP_000855.1|   5-hydroxytryptamine (serotonin) receptor 1...
     ref|NP_000045.1|   arginine vasopressin receptor 2 [Homo sapi...
     ref|NP_064445.1|   opsin 1 (cone pigments), long-wave-sensiti...
     ref|XP_048964.1|   similar to PROBABLE G PROTEIN-COUPLED RECE...
 5   ref|NP_000665.1|   adenosine A1 receptor [Homo sapiens] >gi|1...
     ref|XP_011880.1|   similar to pancreatic polypeptide receptor...
     ref|NP_000676.1|   angiotensin receptor 1; angiotensin recept...
     ref|NP_000674.1|   adrenergic, alpha-2C-, receptor [Homo sapi...
     ref|XP_002705.3|   G protein-coupled receptor 17 [Homo sapiens]
10   ref|NP_000677.1|   angiotensin receptor 2 [Homo sapiens]
     ref|XP_004279.1|   chemokine (C-C motif) receptor 6 [Homo sap...
     ref|NP_000504.1|   opsin 1 (cone pigments), medium-wave-sensi...
     ref|XP_033840.1|   similar to chemokine (C-C motif) receptor ...
     ref|NP_002555.1|   purinergic receptor P2Y, G-protein coupled...
15   ref|XP_006367.1|   purinergic receptor P2Y, G-protein coupled...
     ref|NP_004358.1|   chemokine (C-C motif) receptor 6; chemokin...
     ref|XP_045851.1|   opsin 1 (cone pigments), short-wave-sensit...
     ref|NP_057641.1|   orphan seven-transmembrane receptor, chemo...
     ref|XP_003251.1|   chemokine (C-C motif) receptor 9 [Homo sap...
20   ref|NP_001699.1|   opsin 1 (cone pigments), short-wave-sensit...
     ref|XP_002838.5|   similar to C-C CHEMOKINE RECEPTOR TYPE 11 ...
     ref|NP_000570.1|   chemokine (C-C motif) receptor 5; chemokin...
     ref|NP_006632.2|   chemokine (C-C motif) receptor 9, isoform ...
     ref|NP_000859.1|   5-hydroxytryptamine (serotonin) receptor 2...
25   ref|NP_115942.1|   putative purinergic receptor [Homo sapiens...
     ref|NP_001497.1|   G protein-coupled receptor 32 [Homo sapien...
     ref|NP_061843.1|   G protein-coupled receptor 85; super conse...
     ref|NP_006555.1|   G protein-coupled receptor [Homo sapiens] ...
     ref|NP_065110.1|   cysteinyl leukotriene CysLT2 receptor; cDN...
30   ref|NP_004113.1|   growth hormone secretagogue receptor [Homo...
     ref|NP_055137.1|   opsin 3 (encephalopsin) [Homo sapiens]
     ref|XP_001515.3|   opsin 3 (encephalopsin) [Homo sapiens] >gi...
     ref|NP_005274.1|   G protein-coupled receptor 5 [Homo sapiens...
     ref|NP_061842.1|   super conserved receptor expressed in brai...
35   ref|NP_005291.1|   G protein-coupled receptor 34 [Homo sapien...
     ref|NP_037477.1|   G protein-coupled receptor [Homo sapiens] ...
     ref|XP_003126.1|   chemokine binding protein 2 [Homo sapiens]...
     ref|XP_007392.1|   G protein-coupled receptor 65 [Homo sapiens]
     ref|NP_005284.1|   G protein-coupled receptor 20 [Homo sapiens]
40   ref|NP_005287.1|   G protein-coupled receptor 23 [Homo sapien...
     ref|NP_009195.1|   adrenomedullin receptor; G-protein-coupled...
     ref|NP_003941.1|   coagulation factor II (thrombin) receptor-...
     ref|NP_000701.1|   bradykinin receptor B1 [Homo sapiens]
     ref|NP_000857.1|   5-hydroxytryptamine (serotonin) receptor 1...
45   ref|NP_057652.1|   G-protein coupled receptor SALPR; somatost...
     ref|XP_012745.1|   histamine H4 receptor [Homo sapiens] >gi|1...
     ref|NP_000858.1|   5-hydroxytryptamine (serotonin) receptor 2...
     ref|NP_003599.1|   G protein-coupled receptor 65; T-cell deat...
     ref|NP_001499.1|   G protein-coupled receptor 39 [Homo sapien...
50   ref|XP_007275.2|   bradykinin receptor B1 [Homo sapiens]
     ref|XP_006230.3|   G protein-coupled receptor 72 [Homo sapien...
     ref|XP_037208.1|   histamine receptor H3 [Homo sapiens]
     ref|XP_010168.2|   arginine vasopressin receptor 2 [Homo sapi...
     ref|NP_009163.1|   histamine receptor H3; G protein-coupled r...
55   ref|XP_037209.1|   34432 [Homo sapiens] >gi|14786758|ref|XP_0...
     ref|NP_005273.1|   G protein-coupled receptor 4 [Homo sapiens...
     ref|NP_000668.1|   adenosine A3 receptor [Homo sapiens] >gi|1...
     ref|XP_001499.1|   endothelial differentiation, sphingolipid ...
     ref|NP_114142.1|   G protein-coupled receptor 61 [Homo sapiens]
60   ref|NP_002020.1|   formyl peptide receptor 1 [Homo sapiens] >...
     ref|XP_007108.2|   endothelin receptor type B, isoform 1 [Hom...
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        ref|NP_000106.1|  endothelin receptor type B, isoform 1 [Hom...
        ref|NP_003982.1|  endothelin receptor type B isoform 2 [Homo...
        ref|XP_007276.2|  bradykinin receptor B2 [Homo sapiens] >gi|...
        ref|NP_000614.1|  bradykinin receptor B2 [Homo sapiens] >gi|...
   5    ref|XP_001907.1|  G protein-coupled receptor 25 [Homo sapiens]
        ref|XP_051522.1|  G protein-coupled receptor [Homo sapiens]
        ref|NP_003476.1|  G protein-coupled receptor 68; Ovarian can...
        ref|NP_001498.1|  G protein-coupled receptor 38 [Homo sapien...
        ref|NP_073625.1|  platelet ADP receptor [Homo sapiens] >gi|1...
  10    ref|NP_039226.1|  olfactory receptor, family 10, subfamily H...
        ref|XP_040869.1|  similar to cannabinoid receptor 1 (brain) ...
        ref|NP_004145.1|  pyrimidinergic receptor P2Y, G-protein cou...
        ref|NP_057167.1|  central cannabinoid receptor, isoform a; C...
        ref|NP_062813.1|  seven transmembrane receptor BLTR2; leukot...
  15    ref|NP_149046.1|  olfactory receptor, family 2, subfamily B,...
        ref|NP_000943.1|  platelet-activating factor receptor [Homo ...
        ref|XP_008010.2|  similar to MELANOCYTE STIMULATING HORMONE ...
        ref|NP_063950.1|  olfactory receptor, family 2, subfamily S,...
        ref|NP_001831.1|  central cannabinoid receptor, isoform a; C...
  20    ref|XP_003761.3|  48895 [Homo sapiens] >gi|14724697|ref|XP_0...
        ref|NP_002377.2|  melanocortin 1 receptor (alpha melanocyte ...
        ref|XP_032638.1|  neuromedin U receptor 2 [Homo sapiens]
        ref|NP_005270.1|  G protein-coupled receptor 1 [Homo sapiens]
        ref|NP_005293.1|  G protein-coupled receptor 37 (endothelin ...
  25    ref|NP_004092.1|  coagulation factor II (thrombin) receptor-...
        ref|NP_005289.1|  G protein-coupled receptor 25 [Homo sapiens]
        ref|NP_001727.1|  complement component 5 receptor 1 (C5a lig...
        ref|XP_002667.1|  41743 [Homo sapiens]
        ref|XP_008392.1|  CC chemokine receptor 10 [Homo sapiens] >g...
  30    ref|NP_001496.1|  G protein-coupled receptor 30; chemokine r...
        ref|NP_057686.1|  CC chemokine receptor 10 [Homo sapiens]
        ref|NP_001829.1|  chemokine (C-C motif) receptor 7; Chemokin...
        ref|XP_016412.1|  28082 [Homo sapiens] >gi|14721034|ref|XP_0...
        ref|NP_006009.1|  putative chemokine receptor; GTP-binding p...
  35    ref|NP_000136.1|  follicle stimulating hormone receptor; ova...
        ref|NP_002541.1|  olfactory receptor, family 3, subfamily A,...
        ref|XP_002212.2|  follicle stimulating hormone receptor [Hom...
        ref|NP_004769.1|  G protein-coupled receptor 44; chemoattrac...
        ref|XP_015921.1|  similar to putative chemokine receptor; GT...
  40    ref|XP_015923.1|  putative chemokine receptor; GTP-binding p...
        ref|NP_055380.1|  olfactory receptor, family 1, subfamily A,...
        ref|NP_065133.1|  putative G protein-coupled receptor 92 [Ho...
        ref|XP_008716.1|  melanocortin 4 receptor [Homo sapiens]
        ref|NP_039225.1|  olfactory receptor, family 11, subfamily A...
  45    ref|NP_002556.1|  pyrimidinergic receptor P2Y, G-protein cou...
        ref|NP_005903.1|  melanocortin 4 receptor [Homo sapiens]
        ref|NP_005297.1|  G protein-coupled receptor 43 [Homo sapien...
        ref|XP_012667.1|  olfactory receptor, family 1, subfamily A,...
        ref|NP_112221.1|  olfactory receptor, family 12, subfamily D...
  50    ref|NP_039227.1|  olfactory receptor, family 10, subfamily H...
        ref|NP_059976.1|  olfactory receptor, family 7, subfamily C,...
        ref|NP_005279.1|  G protein-coupled receptor 12 [Homo sapien...
        ref|NP_001948.1|  endothelin receptor type A [Homo sapiens]
        ref|NP_005904.1|  melanocortin 5 receptor [Homo sapiens] >gi...
  55    ref|NP_110401.1|  prostate specific G-protein coupled recept...
        ref|NP_004758.1|  endothelin type b receptor-like protein 2 ...
        ref|NP_003544.1|  olfactory receptor, family 1, subfamily E,...
        ref|NP_000520.1|  melanocortin 2 receptor; Melanocortin-2 re...
        ref|NP_036492.1|  olfactory receptor, family 1, subfamily F,...
  60    ref|NP_002557.1|  purinergic receptor P2Y, G-protein coupled...
        ref|XP_030219.1|  gonadotropin-releasing hormone receptor [H...
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        ref|XP_009029.4|  purinergic receptor P2Y, G-protein coupled...
        ref|NP_036284.1|  endothelial cell differentiation gene 7; c...
        ref|NP_039228.1|  olfactory receptor, family 10, subfamily H...
        ref|NP_036484.1|  olfactory receptor, family 1, subfamily A,...
   5    ref|XP_012668.1|  olfactory receptor, family 1, subfamily A,...
        ref|NP_001983.1|  coagulation factor II receptor precursor; ...
        ref|NP_036501.1|  olfactory receptor, family 2, subfamily F,...
        ref|NP_055694.1|  putative G-protein-coupled receptor; G pro...
        ref|XP_004852.1|  olfactory receptor, family 2, subfamily F,...
  10    ref|NP_000949.1|  prostaglandin E receptor 4 (subtype EP4) [...
        ref|NP_072093.1|  putative leukocyte platelet-activating fac...
        ref|NP_110503.1|  olfactory receptor, family 5, subfamily V ...
        ref|XP_003907.1|  coagulation factor II receptor precursor [...
        ref|XP_004216.1|  similar to olfactory receptor 89 (H. sapie...
  15    ref|NP_112167.1|  olfactory receptor, family 2, subfamily J,...
        ref|NP_002542.1|  olfactory receptor, family 3, subfamily A,...
        ref|NP_036505.1|  olfactory receptor, family 3, subfamily A,...
        ref|NP_005292.1|  G protein-coupled receptor 35 [Homo sapiens]
        ref|NP_004045.1|  complement component 3a receptor 1; comple...
  20    ref|NP_005290.1|  G protein-coupled receptor 31 [Homo sapiens]
        ref|NP_005233.2|  coagulation factor II (thrombin) receptor-...
        ref|NP_003546.1|  olfactory receptor, family 1, subfamily G,...
        ref|XP_003671.3|  coagulation factor II (thrombin) receptor-...
        ref|NP_112163.1|  olfactory receptor, family 7, subfamily A,...
  25    ref|NP_039229.1|  olfactory receptor, family 10, subfamily C...
        ref|NP_002539.1|  olfactory receptor, family 1, subfamily D,...
        ref|XP_037263.1|  similar to coagulation factor II (thrombin...
        ref|NP_067647.1|  leucine-rich repeat-containing G protein-c...
        ref|NP_065103.1|  inflammation-related G protein-coupled rec...
  30    ref|NP_055314.1|  putative purinergic receptor [Homo sapiens...
        ref|NP_036509.1|  olfactory receptor, family 7, subfamily C,...
        ref|NP_036507.1|  olfactory receptor, family 52, subfamily A...
        ref|XP_035507.1|  similar to NONE_RETURNED (H. sapiens) [Hom...
        ref|XP_004280.1|  G protein-coupled receptor 31 [Homo sapiens]
  35    ref|NP_003545.1|  olfactory receptor, family 1, subfamily B,...
        ref|XP_036497.1|  olfactory receptor, family 1, subfamily F,...
        ref|NP_061844.1|  super conserved receptor expressed in brai...
        ref|NP_063941.1|  melanocortin 3 receptor [Homo sapiens]
        ref|NP_009091.1|  olfactory receptor, family 2, subfamily H,...
  40    ref|XP_008678.2|  olfactory receptor, family 1, subfamily D,...
        ref|NP_003543.1|  olfactory receptor, family 1, subfamily D,...
        ref|XP_011731.3|  similar to adrenergic, beta-3-, receptor (...
        ref|XP_009545.1|  melanocortin 3 receptor [Homo sapiens]
        ref|NP_076403.1|  G protein-coupled receptor 86 [Homo sapiens]
  45    ref|XP_042200.1|  G protein-coupled receptor 86 [Homo sapiens]
        ref|NP_004711.2|  endothelial differentiation, lysophosphati...
        ref|NP_037440.1|  platelet activating receptor homolog [Homo...
        ref|NP_005217.1|  endothelial differentiation, sphingolipid ...
        ref|NP_005295.1|  G protein-coupled receptor 41 [Homo sapien...
  50    ref|NP_005296.1|  G protein-coupled receptor 42 [Homo sapiens]
        ref|NP_115943.1|  putative chemokine receptor [Homo sapiens]...
        ref|NP_055381.1|  olfactory receptor, family 1, subfamily D,...
        ref|XP_042826.1|  luteinizing hormone/choriogonadotropin rec...
        ref|XP_010797.3|  luteinizing hormone/choriogonadotropin rec...
  55    ref|NP_000224.1|  luteinizing hormone/choriogonadotropin rec...
```

Table 9

>G(S)-1
mgclgnskte dqrneekaqr eankkiekql qkdkqvyrat hrllllgage sgkstivkqm

```
    rilhvngfng  eggeedpqaa  rsnsdgekat  kvqdiknnlk  eaietivaam  snlvppvela
    npenqfrvdy  ilsvmnvpdf  dfppefyeha  kalwedegvr  acyersneyq  lidcaqyfld
    kidvikqady  vpsdqdllrc  rvltsgifet  kfqvdkvnfh  mfdvggqrde  rrkwiqcfnd
    vtaiifvvas  ssynmvired  nqtnrlqeal  nlfksiwnnr  wlrtisvilf  lnkqdllaek
5   vlagkskied  yfpefarytt  pedatpepge  dprvtrakyf  irdeflrist  asgdgrhycy
    phftcavdte  nirrvfndcr  diiqrmhlrq  yell (SEQ ID NO:13).


        >G(S)-2
    mgclgnskte  dqrneekaqr  eankkiekql  qkdkqvyrat  hrllllgage  sgkstivkqm
10  rilhvngfng  eggeedpqaa  rsnsdgseka  tkvqdiknnl  keaietivaa  msnlvppvel
    anpenqfrvd  yilsvmnvpd  fdfppefyeh  akalwedegv  racyersney  qlidcaqyfl
    dkidvikqad  yvpsdqdllr  crvltsgife  tkfqvdkvnf  hmfdvggqrd  errkwiqcfn
    dvtaiifvva  sssynmvire  dnqtnrlqea  lnlfksiwnn  rwlrtisvil  flnkqdllae
    kvlagsksie  dyfpefaryt  tpedatpepg  edprvtraky  firdeflris  tasgdgrhyc
15  yphftcavdt  enirrvfndc  rdiiqrmhlr  qyell (SEQ ID NO:14).


        >G(S)-3
    mgclgnskte  dqrneekaqr  eankkiekql  qkdkqvyrat  hrllllgage  sgkstivkqm
    rilhvngfng  dekatkvqdi  knnlkeaiet  ivaamsnlvp  pvelanpenq  frvdyilsvm
20  nvpdfdfppe  fyehakalwe  degvracyer  sneyqlidca  qyfldkidvi  kqadyvpsdq
    dllrcrvlts  gifetkfqvd  kvnfhmfdvg  gqrderrkwi  qcfndvtaii  fvvasssynm
    virednqtnr  lqealnlfks  iwnnrwlrti  svilflnkqd  llaekvlagk  skiedyfpef
    aryttpedat  pepgedprvt  rakyfirdef  lristasgdg  rhycyphftc  avdtenirrv
    fndcrdiiqr  mhlrqyell (SEQ ID NO:15).
25

        >G(S)-4
    mgclgnskte  dqrneekaqr  eankkiekql  qkdkqvyrat  hrllllgage  sgkstivkqm
    rilhvngfng  dsekatkvqd  iknnlkeaie  tivaamsnlv  ppvelanpen  qfrvdyilsv
    mnvpdfdfpp  efyehakalw  edegvracye  rsneyqlidc  aqyfldkidv  ikqadyvpsd
30  qdllrcrvlt  sgifetkfqv  dkvnfhmfdv  ggqrderrkw  iqcfndvtai  ifvvasssyn
    mvirednqtn  rlqealnlfk  siwnnrwlrt  isvilflnkq  dllaekvlag  kskiedyfpe
    faryttpeda  tpepgedprv  trakyfirde  flristasgd  grhycyphft  cavdtenirr
    vfndcrdiiq  rmhlrqyell (SEQ ID NO:16).


35      >G(s)-xl
    meisgppfei  gsapagvddt  pvnmdsppia  ldgppikvsg  apdkreraer  ppveeeaaem
    egaadaaegg  kvpspgygsp  aagaasadta  araapaapad  pdsgatpedp  dsgtapadpd
    sgafaadpds  gaapaapadp  dsgaapdapa  dpdsgaapda  padpdagaap  eapaapaaae
    traahvapaa  pdagaptapa  asatraaqvr  raasaapasg  arrkihlrpp  speiqaadpp
40  tprptrasaw  rgksessrgr  rvyydegvas  sdddssgdes  ddgtsgclrw  fqhrrnrrrr
    kpqrnllrnf  lvqafggcfg  rsespqpkas  rslkvkkvpl  aekrrqmrke  alekraqkra
    ekkrsklidk  qlqdekmgym  cthrllll (SEQ ID NO:17).


        >g-olf
45  mgclggnskt  tedqgvdeke  rreankkiek  qlqkerlayk  athrllllga  gesgkstivk
    qmrilhvngf  npeekkqkil  dirknvkdai  vtivsamsti  ippvplanpe  nqfrsdyiks
    iapitdfeys  qeffdhvkkl  wddegvkacf  ersneyqlid  caqyflerid  svslvdytpt
    dqdllrcrvl  tsgifetrfq  vdkvnfhmfd  vggqrderrk  wiqcfndvta  iiyvaacssy
    nmviredrnt  nrlresldlf  esiwnnrwlr  tisiilflnk  qdmlaekvla  gkskiedyfp
50  eyanytvped  atpdagedpk  vtrakffird  lflristatg  dgkhycyphf  tcavdtenir
    rvfndcrdii  qrmhlkqyel  l (SEQ ID NO:18).


        >Il
    mgctlsaedk  aaverskmid  rnlredgeka  arevkllllg  agesgkstiv  kqmkiiheag
55  yseeeckqyk  avvysntiqs  iiaiiramgr  lkidfgdsar  addarqlfvl  agaaeegfmt
    aelagvikrl  wkdsgvqacf  nrsreyqlnd  saayylndld  riaqpnyipt  qqdvlrtrvk
    ttgivethft  fkdlhfkmfd  vggqrserkk  wihcfegvta  iifcvalsdy  dlvlaedeem
    nrmhesmklf  dsicnnkwft  dtsiilflnk  kdlfeekikk  splticypey  agsntyeeaa
```

ayiqcqfedl nkrkdtkeiy thftcatdtk nvqfvfdavt dviiknnlkd cglf (SEQ ID NO:19).

> I2

mgctvsaedk aaaerskmid knlredgeka arevkllllg agesgkstiv kqmkiihedg
yseeecrqyr avvysntiqs imaivkamgn lqidfadpsr addarqlfal sctaeeqgvl
pddlsgvirr lwadhgvqac fgrsreyqln dsaayylndl eriaqsdyip tqqdvlrtrv
kttgivethf tfkdlhfkmf dvggqrserk kwihcfegvt aiifcvalsa ydlvlaedee
mnrmhesmkl fdsicnnkwf tdtsiilfln kkdlfeekit hsplticfpe ytgankydea
asyiqskfed lnkrkdtkei ythftcatdt knvqfvfdav tdviiknnlk dcglf (SEQ ID NO:20).

> I3

mgctlsaedk aaverskmid rnlredgeka akevkllllg agesgkstiv kqmkiihedg
ysedeckqyk vvvysntiqs iiaiiramgr lkidfgeaar addarqlfvl agsaeegvmt
pelagvikrl wrdggvqacf srsreyqlnd sasyylndld risqsnyipt qqdvlrtrvk
ttgivethft fkdlyfkmfd vggqrserkk wihcfegvta iifcvalsdy dlvlaedeem
nrmhesmklf dsicnnkwft etsiilflnk kdlfeekikr splticypey tgsntyeeaa
ayiqcqfedl nrrkdtkeiy thftcatdtk nvqfvfdavt dviiknnlke cgly (SEQ ID NO:21).

>G01

mgctlsaeer aalerskaie knlkedgisa akdvkllllg agesgkstiv kqmkiihedg
fsgedvkqyk pvvysntiqs laaivramdt lgieygdker kadakmvcdv vsrmedtepf
saellsammr lwgdsgiqec fnrsreyqln dsakyyldsl drigaadyqp teqdilrtrv
kttgivethf tfknlhfrlf dvggqrserk kwihcfedvt aiifcvalsg ydqvlhedet
tnrmheslml fdsicnnkff idtsiilfln kkdlfgekik ksplticfpe ytgpntyeda
aayiqaqfes knrspnkeiy chmtcatdtn niqvvfdavt diiiannlrg cgly (SEQ ID NO:22).

>g02

mgctvsaedk aaaerskmid knlredgeka arevkllllg agesgkstiv kqmkiihedg
yseeecrqyr avvysntiqs imaivkamgn lqidfadpsr addarqlfal sctaeeqgvl
pddlsgvirr lwadhgvqac fgrsreyqln dsaayylndl eriaqsdyip tqqdvlrtrv
kttgivethf tfkdlhfkmf dvggqrserk kwihcfegvt aiifcvalsa ydlvlaedee
mnrmhesmkl fdsicnnkwf tdtsiilfln kkdlfeekit hsplticfpe ytgankydea
asyiqskfed lnkrkdtkei ythftcatdt knvqfvfdav tdviiknnlk dcglf (SEQ ID NO:23).

>G(T-1)

mgagasaeek hsrelekklk edaekdartv klllllgages gkstivkqmk iihqdgysle
eclefiaiiy gntlqsilai vramttlniq ygdsarqdda rklmhmadti eegtmpkems
diiqrlwkds giqacferas eyqlndsagy ylsdlerlvt pgyvpteqdv lrsrvkttgi
ietqfsfkdl nfrmfdvggq rserkkwihc fegvtciifi aalsaydmvl veddevnrmh
eslhlfnsic nhryfattsi vlflnkkdvf fekikkahls icfpdydgpn tyedagnyik
vqflelnmrr dvkeiyshmt catdtqnvkf vfdavtdiii kenlkdcglf (SEQ ID NO:24).

>G(T-2)

mgsgasaedk elakrskele kklqedadke aktvkllllg agesgkstiv kqmkiihqdg
yspeeclefk aiiygnvlqs ilaiiramtt lgidyaepsc addgrqlnnl adsieegtmp
pelvevirrl wkdggvqacf eraaeyqlnd sasyylnqle ritdpeylps eqdvlrsrvk
ttgiietkfs vkdlnfrmfd vggqrserkk wihcfegvtc iifcaalsay dmvlveddev
nrmheslhlf nsicnhkffa atsivlflnk kdlfeekikk vhlsicfpey dgnnsyddag
nyiksqfldl nmrkdvkeiy shmtcatdtq nvkfvfdavt diiikenlkd cglf (SEQ ID NO:25).

>G(Z)

mvflsgnasd ssnctqppap vniskaillg vilgglilfg vlgnilvils vachrhlhsv
thyyivnlav adllltstvl pfsaifevlg ywafgrvfcn iwaavdvlcc tasimglcii
sidryigvsh plryptivtq rrglmallcv walslvisig plfgwrqpap edeticqine
epgyvlfsal gsfylplaii lvmycrvyvv akresrglks glktdksdse qvtlrihrkn
apaggsgmas aktkthfsvr llkfsrekka aktlgivvgc fvlcwlpffl vmpigsffpd

```
          fkpsetvfki  vfwlgylnsc  inpiiypcss  qefkkafqnv  lriqclcrkq  sskhalgytl
          hppsqavegq  hkdmvripvg  sreafygisr  tdgvcewkff  ssmprgsari  tvskdqssct
          tarvrsksfl  qvcccvepst  psldknhqvp  tikvhtisls  engeev (SEQ ID NO:26).
```

5   >G(Q)
```
          macclseeak  earrindeie  rqlrrdkrda  rrelkllllg  tgesgkstfi  kqmriihgsg
          ysdedkrgft  klvyqnifta  mqamiramdt  lkipykyehn  kahaqlvrev  dvekvsafen
          pyvdaikslw  ndpgiqecyd  rrreyqlsds  tkyylndldr  vadpaylptq  qdvlrvrvpt
          tgiieypfdl  qsvifrmvdv  ggqrserrkw  ihcfenvtsi  mflvalseyd  qvlvesdnen
          rmeeskalfr  tiitypwfqn  ssvilflnkk  dlleekimys  hlvdyfpeyd  gpqrdaqaar
          efilkmfvdl  npdsdkiiys  hftcatdten  irfvfaavkd  tilqlnlkey  nav (SEQ ID NO:27).
```

>G(Y-11)
```
          mtlesmmacc  lsdevkeskr  inaeiekqlr  rdkrdarrel  klllllgtges  gkstfikqmr
          iihgagysee  dkrgftklvy  qniftamqam  irametlkil  ykyeqnkana  llirevdvek
          vttfehqyvs  aiktlwedpg  iqecydrrre  yqlsdsakyy  ltdvdriatl  gylptqqdvl
          rvrvpttgii  eypfdlenii  frmvdvggqr  serrkwihcf  envtsimflv  alseydqvlv
          esdnenrmee  skalfrtiit  ypwfqnssvi  lflnkkdlle  dkilyshlvd  yfpefdgpqr
          daqaarefil  kmfvdlnpds  dkiiyshftc  atdtenirfv  faavkdtilq  lnlkeynlv (SEQ ID NO:28)
```

20
>G(Y-12)
```
          msgvvrtlsr  cllpaeagga  rerragsgar  daerearrrs  rdidallare  rravrrlvki
          lllgagesgk  stflkqmrii  hgrefdqkal  lefrdtifdn  ilkgsrvlvd  ardklgipwq
          ysenekhgmf  lmafenkagl  pvepatfqly  vpalsalwrd  sgireafsrr  sefqlgesvk
          yfldnldrig  qlnyfpskqd  illarkatkg  ivehdfvikk  ipfkmvdvgg  qrsqrqkwfq
          cfdgitsilf  mvssseydqv  lmedrrtnrl  vesmnifeti  vnnklffnvs  iilflnkmdl
          lvekvktvsi  kkhfpdfrgd  phqledvqry  lvqcfdrkrr  nrskplfhhf  ttaidtenvr
          fvfhavkdti  lqenlkdiml  q (SEQ ID NO:29).
```

30 >G(Y-13
```
          madflpsrsv  lsvcfpgcll  tsgeaeqqrk  skeidkclsr  ektyvkrlvk  illlgagesg
          kstflkqmri  ihgqdfdqra  reefrptiys  nvikgmrvlv  dareklhipw  gdnsnqqhgd
          kmmsfdtrap  maaqgmvetr  vflqylpair  alwadsgiqn  aydrrrefql  gesvkyfldn
          ldklgepdyi  psqqdillar  rptkgiheyd  feiknvpfkm  ldvggqrser  krwfecfdsv
35        tsilflvsss  efdqvlmedr  ltnrltesln  ifetivnnrv  fsnvsiilfl  nktdlleekv
          qivsikdyfl  efegdphclr  dvqkflvecf  rnkrrdqqqk  plyhhfttai  ntenirlvfr
          dvkdtilhdn  lkqlmlq (SEQ ID NO:30).
```

>G(Y-14)
40
```
          magcccsae   ekesqrisae  ierqlrrdkk  darrelklll  lgtgesgkst  fikqmriihg
          sgysdedrkg  ftklvyqnif  tamqamiram  dtlriqyvce  qnkenaqiir  evevdkvsml
          sreqveaikq  lwqdpgiqec  ydrrreyqls  dsakyyltdi  driatpsfvp  tqqdvlrvrv
          pttgiieypf  dleniifrmv  dvggqrserr  kwihcfesvt  siiflvalse  ydqvlaecdn
          enrmeeskal  fktiitypwf  lnssvilfln  kkdlleekim  yshlisyfpe  ytgpkqdvra
45        ardfilklyq  dqnpdkekvi  yshftcatdt  dnirfvfaav  kdtilqlnlr  efnlv (SEQ ID NO:31).
```

>G(Y-15)
```
          marsltwrcc  pwcltedeka  aarvdqeinr  illeqkkqdr  gelkllllgp  gesgkstfik
          qmriihgagy  seeerkgfrp  lvyqnifvsm  ramieamerl  qipfsrpesk  hhaslvmsqd
50        pykvttfekr  yaaamqwlwr  dagiracyer  rrefhlldsa  vyylshleri  teegyvptaq
          dvlrsrmptt  gineycfsvq  ktnlrivdvg  gqkserkkwi  hcfenviali  ylaslseydq
          cleennqenr  mkeslalfgt  ilelpwfkst  svilflnktd  ileekiptsh  latyfpsfqg
          pkqdaeaakr  fildmytrmy  tgcvdgpegs  kkgarsrrlf  shytcatdtq  nirkvfkdvr
          dsvlarylde  inll (SEQ ID NO:32).
```

55
>Gusducin
```
          mfdvggqrse  rkkwihcfeg  vtciifcaal  saydmvlved  eevnrmhesl  hlfnsicnhk
          yfsttsivlf  lnkkdifqek  vtkvhlsicf  peytgpntfe  dagnyiknqf  ldlnlkkedk
```

```
eiyshmtcat dtqnvkfvfd avtdiiiken lkdcglf (SEQ ID NO:33).
```

1. Selection of nucleic acid sensor molecules which are activated by GPCRs not bound to ligand:

5   A library of up to 10^{17} variants of *in vitro* synthesized ribozymes is allowed to react with purified GPCRs at a final concentration of luM GPCR. Selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in prior examples. Selections are carried out in parallel fashion. Selections can also be carried out in mixed pools of anywhere from 5-10 GPCRs. In the final rounds of nucleic acid sensor molecule selection,

10   the RNA pools may separated into aliquots which may then be used to carry out *in vitro* selection against single GPCR proteins to yield unique nucleic acid sensor molecules selective for all 400 plus GPCRs.

2. Selection of nucleic acid sensor molecules which are activated by ligand bound GPCRs.

15   Stable complexes of each GPCR and corresponding -ligand are formed with from 1-10 equivalents of ligand. Selection of catalytic nucleic acid sensor molecules is carried out by procedures outlined in the Detailed Description. Selections are carried out in parallel fashion. Selections can also be carried out in mixed pools of anywhere from 5-10 GPCR-ligand complexes. In the final rounds of nucleic acid sensor molecule selection, the RNA

20   pools may separated into aliquots which may then be used to carry out *in vitro* selection against single GPCR-ligand complexes to yield unique nucleic acid sensor molecules selective for all GPCR-ligand complexes.

         Selection of nucleic acid sensor molecules using peptide fragments of GPCRs Nucleic acid sensor molecules that specifically recognize conformational isoforms of

25   GPCRs that are revealed upon ligand binding can also be selected for using the methods described herein.

         Molecular cloning studies have identified over 400 human GPCRs, and have identified the ligands for 120. GPCRs consist of three domains: an extracellular N-terminus, a central domain of seven trans-membrane helices, and a cytoplasmic C-terminus.

30   Activation of GPCRs is induced by ligand binding, which causes a conformational change in the receptor transmitting a signal across the plasma membrane to intracellular members of a signaling pathway. This method provides for generation of unique biosensors for each GPCR.

A library of up to 10^{17} variants of *in vitro* synthesized ribozymes is allows to react with peptide fragments of the GPCRs comprising regions of the GPCR that are exposed upon activation. Nucleic acid sensor molecules which recognize these domains are then capable of recognizing them within the context of the full length protein and hence

5   recognize the activated state of the GPCR. Examples of the use of peptide fragments to generate nucleic acid sensor molecules which recognize the full length protein are known in the art and are incorporated herein. See, for example, data on nucleic acid sensor molecule selection and recognition of HIV rev peptide and full length protein [Michael Robertson, 2001, University of Texas, Austin, Ph.D. Dissertation]. In the case of HIV rev, unique

10   peptide sequences are recognized both as free peptides and in the context of the full protein.

Nucleic acid sensor molecules specific for GPCRs are generated by *in vitro* selection for recognition of peptide fragments of the GPCRs comprising regions of the GPCR that are exposed to the inside face of the plasma membrane when ligand binds to the GPCR. Exemplary suitable GPCR peptide fragments are presented in Table 9. Nucleic acid sensor

15   molecules which recognize these peptides are then capable of recognizing them within the context of the full length protein and hence recognize the activated state of the GPCR.

Example 6. Nucleic acid sensor molecule specific to phosphodiesterase (PDE) for target validation

20   Nucleic acid sensor molecules are generated to be specific to various subclasses of PDEs are used for understanding the role of PDE subclasses in the molecular pathology of disease, and as PDE target validation. For example, nucleic acid sensor molecules which are modulated by each of four PDE4 subtypes have specific utility in understanding the role

25   of PDE4 in human disease. The four subclasses of PDE4 are differentially localized between cell type and also the PDE4 isozymes differ with respect to their intracellular localization. This differential localization, together with the transcriptional regulation and post-translational modification, controls the cAMP level in cells in response to the cells' environment (Muller, Engels et al. 1996).

30   The cDNAs for four PDE4 subtypes are cloned from human blood leukocyte cDNA library as described (Wang, Myers et al. 1997). Each subclass of PDE4 is expressed as recombinant protein fused with a His-tag in *E. coli* or SF9 insect cells (Richter, Hermsdorf et al. 2000) (Wang, Myers et al. 1997). The expressed proteins are purified through Ni^{++} columns according to established procedures. Catalytic nucleic acid sensor molecules

35   modulated by the four subclasses of PDE4 are then selected as described above. The

nucleic acid sensor molecules are tested for their subclass specificity, by determining the switch factor.

Tissue samples from different organs can be prepared, and the cell extract can be tested against a panel of PDE4 subclass-specific nucleic acid sensor molecules to determine
5   the protein level of each PDE in the organ. Thus, one can obtain more precise information about PDE4 levels relative to methods based on measuring the mRNA level (Bloom and Beavo 1996) (Obernolte, Ratzliff et al. 1997) (Nagaoka, Shirakawa et al. 1998).

Likewise, different classes of PDE (PDE1-11) are expressed in a tissue-specific manner and play different physiological roles (Conti 2000), and the subcellular localization
10   of PDE regulates their activity. Accordingly, the nucleic acid sensor molecule can be used to determine the subcellular localization of each PDE from fractionated cell extracts (Bolger, Erdogan et al. 1997), or in situ hybridization technique (Sirinarumitr, Paul et al. 1997).

The nucleotide sequences of a cAMP-dependent PDE nucleic acid sensor molecule
15   and cGMP-dependent nucleic acid sensor molecule are presented in Table 10. Allosteric domains are shown in bold font and the cleavage site nucleotide is underlined. CGMP modulated NASMs that are configured for homogeneous, solution based fluorescence assays (FRET) are shown the Figure 62. Multiplexed camp and cGMP-modulated FRET-sensor NASM-based assays are shown in Figure 65B. The optical NASMs modulated by
20   cAMP and cGMP are used in PDE assays as described in detail below.

Table 10

cAMP-Hammerhead RNA seq:

25   5'- GGGC GAC CC UGA UGA GCC UGU GGA AAC AGA CGU GGC ACA UGA CUA CGU CGA
     AAC GGU GAA AGC CGU AGG UUG   CCC -3' (SEQ ID NO:34)

cGMP-Hammerhead RNA seq:
30

5'- GGGC GAC CC UGA UGA GCC CUG CGA UGC AGA AAG GUG CUG ACG ACA CAU CGA
     AAC GGU GAA AGC CGU AGG UUG   CCC -3' (SEQ ID NO:35)

The cAMP and cGMP-dependent nucleic acid sensor molecules were added to a
35   solution containing various amounts of PDE and the corresponding cyclic nucleotide (cAMP or cGMP). A decreasing amount of the cyclic nucleotide was found to correspond to the increasing amount of PDE. These results demonstrate that the cyclic nucleotide-dependent nucleic acid sensor molecules can be used to measure PDE activity.

The calmodulin activated PDE specific for cGMP was used under the following enzyme assay conditions: Assay conditions (10 mM Tris, 20 mM MgCl$_2$ 100 μM CaCl$_2$) 200 nM cGMP-NASM; the assay is quenched with 0.1% SDS and then 200 nM cGMP-NASM is added to the mixture. Remaining cGMP was determined by the amount of

5 conversion of nucleic acid sensor molecule to product using a gel based radioactive product-release (gel-shift) assay format. The nucleic acid sensor molecule is active in a variety of formats and is not inhibited by GMP produced by the components of the PDE assay. Similarly, the cAMP nucleic acid sensor molecule is active in a variety of assay formats and is not inhibited by GMP produced by the components of the PDE assay. The conversion of

10 cGMP to GMP is followed by the optical NASM formats described in Figures 62-72.

High Throughput Screening (HTS) assays using cAMP-dependent PDE nucleic acid sensor molecules:

A cAMP-dependent nucleic acid sensor molecule is used in HTS assays for PDEs

15 (PDE1, PDE2, PDE3, PDE4, PDE7, PDE8, PDE10, and PDE11). Similarly, cGMP-dependent-nucleic acid sensor molecules can be used in HTS assays for PDEs (PDE5, PDE9, PDE10, and PDE11). Representative cAMP-dependent and cGMP-dependent PDE nucleic acid sensor molecules are shown above, and in Figures 62-72 for all solution and chip-based NASM assay configurations.

20 Each class of PDE can be isolated from human tissue (Ballard, Gingell et al. 1998), or expressed as recombinant proteins in various system (*e.g. E coli*, SF9 cells). Thus, the nucleic acid sensor molecule monitors the PDE activity in the presence and the absence of candidate drugs. For example, PDE and its substrate (*i.e.*, cAMP and/or cGMP) are incubated at predetermined durations in a multiwell chamber (*e.g.*, 96, 384 well) with

25 various concentration of compounds for screening, and the reaction is terminated by changing the buffer conditions (*e.g.*, addition of sufficient amount of EGTA, shifting buffer pH), or by separating enzyme and substrate (*e.g.*, filtration). Next, the nucleic acid sensor molecules are added to measure the altered concentration of the substrate, cAMP and cGMP. Alternatively, optical nucleic acid sensor molecules can be added without

30 terminating the PDE activity. CAMP and cGMP modulated these HTS assays, as described in Figure 62 and Figure 63.

cAMP- or cGMP-dependent nucleic acid sensor molecules can also be used to characterize the IC50 of the drug *in vitro*. A PDE assay is performed with serial dilutions of a compound of interest. Purified PDE or, alternatively, soluble extract from cells

(Moreland, Goldstein et al. 1998) can be used for the assay. The assay can be performed as described herein.

Alternatively, cAMP- or cGMP-dependent nucleic acid sensor molecules (Figures 62, 33, and 64) are used to characterize the IC50 values of drug candidate *in vitro* by

5 analyzing cAMP- or cGMP synthesized by adenyl and guanyl cyclases. Adenylate and guanylate cyclase assays are set up with serial dilution of a compound of interest. Membrane fractions containing adenylate and guanylate cyclases are used for the assay. The assay can be setup as described in the literature using ATP or GTP as the substrate. *Competitive assays using PDE nucleic acid sensor molecules:*

10 Nucleic acid sensor molecules are generated that interact with the active sites of PDEs. PDE4 proteins are obtained as described above. The nucleic acid sensor molecules are selected against PDE4 with negative selection in the presence of PDE4 complexed with subnanomolar inhibitor (*e.g.*, Rolipram). Thus, the nucleic acid sensor molecule is modulated by free, uncomplexed PDE4, the PDE nucleic acid sensor molecules compete for

15 PDE binding with inhibitors.

The direct inhibition by the nucleic acid sensor molecules can be tested using commercially available PDE assay kits (Amersham SPA assay kit for cAMP, Molecular Devices HEEP cAMP assay kit). In drug screening, the competition is performed by monitoring the signal from the nucleic acid sensor molecules in the presence of various

20 inhibitors. Purified PDE or soluble cell extract from appropriate source (*e.g.*, Wistar rat brain (Andersson, Gemalmaz et al. 1999)) is incubated with nucleic acid sensor molecules (100 nM) in the presence and the absence of compounds in 10 mM Tris buffer pH 7.5 containing 10 mM MgCl$_2$. The changes in the initial rate of each nucleic acid sensor molecule response in the presence and the absence of the drug can be monitored in

25 homologous system. Multiple PDEs can be tested against a same compound in the same well. This assay is expanded if desired to determine the tissue specific interaction of each class of PDE and any compounds.

Cell-based assays using cyclic nucleotide-dependent PDE nucleic acid sensor molecules

30 Nucleic acid sensor molecules are used to monitor the cellular cAMP and cGMP level in response to the injection of drugs in tissue or rat cell lines. For example, strips of human corpus collasum (HCC) tissue or rat HCC cell lines (N1S1 and McA-RH7777 cells) can be incubated in the presence and absence of a drug against PDE5 (Min, Kim et al. 2000) (Arora, de Groen et al. 1996), and the cGMP specific nucleic acid sensor molecule can be

used to measure the amount of cGMP in soluble extract from the tissue or cell sample as described above.

Alternatively, the cAMP, and cGMP-dependent nucleic acid sensor molecules are incorporated into a reporter-gene plasmid as described above. This construct is introduced

5   in cell lines by standard transfection (e.g. lipid-mediated transfection, calcium-phosphate co-precipitation, microinjection, electroporation, retroviral infection). The level of cGMP or camp in the cell is measured by the expression of the reporter gene.

Class specific PDE assay:

10   Nucleic acid sensor molecules are selected for the catalytic domains of each class of PDE1-11 are prepared. These nucleic acid sensor molecules are then used for target validation as described above.

Alternatively, the nucleic acid sensor molecules are used in competitive inhibition assays. Competitive nucleic acid sensor molecules are used in *in vitro* assays to screen

15   compounds against multiple PDEs in multiplex assays, as described above.

Example 7. Pharmacokinetics studies using nucleic acid sensor molecules

Nucleic acid sensor molecules modulated by drug leads or drug compounds used in preclinical testing and clinical trial for pharmacokinetics studies are selected and identified

20   as described in the Detailed Description. A human serum sample with or without the administration of a drug or other therapeutic agent is prepared (Berzas Nevado, Rodriguez Flores et al. 2001). The nucleic acid sensor molecule is added to the sample. The nucleic acid sensor molecule is then used in optical or PCR based detection methods as described in later examples, thereby quantifying the drug concentration in the whole serum or extract

25   from the serum.

The nucleic acid sensor molecule modulated by various drugs or leads can also be used to determine the drug distribution in an animal model system. For example, a drug can be administrated in animals (e.g., Sprague Dawley rats, New Zealand white rabbit) by IV or orally (Andersson, Gemalmaz et al. 1999) (Jeremy, Ballard et al. 1997). At various time

30   intervals after drug administration, the animal is sacrificed. Various organs are tested for the drug distribution by in situ hybridization using the drug-dependent nucleic acid sensor molecule. Alternatively, each organs/serum is prepared for pharmacokinetic studies.

Example 8. Cell-permeability studies using nucleic acid sensor molecules

Nucleic acid sensor molecules against a test compound are used to test cell permeability of the compound. These nucleic acid sensor molecules can be incorporated into a reporter gene construct, if desired, to make a drug-sensitive reporter gene system as described above. This construct is introduced in established cell lines (*e.g.* HELA cells, 293

5 cell, CHO cell). The cells are cultured in various concentrations of drug in media, and the expression of the reporter gene is monitored.

Example 9. Expression and purification of recombinant MAPKs

All protein ORFs were obtained from Upstate Biotech, and cloned into pRSET

10 (Invitrogen), except for ERK which was cloned directly downstream from a hexahistidine tag into pRSET from which the leader sequence had been removed. Constructs were sequence verified before transformation into Rosetta plys(S) cells (Novagen). After growth and induction as described below, cells were washed in 10 ml PBS per liter of culture and resuspended in 7.5 ml Lysis Buffer (500 mM KCl, 20 mM Tris-Cl, pH 8.0, 10% glycerol,

15 0.5% NP-40, supplemented with 1 Complete EDTA-Free Protease Inhibitor tablet (Roche) per 50 ml) per liter of culture. Cells were frozen in liquid nitrogen and stored at -80 °C. Lysis and clarification was accomplished by rapid thawing in a 40 °C bath for 10 minutes, incubation at 4 °C for thirty minutes, and centrifugation for 60 minutes at 100,000 x g. As a standard first step, metal chelate affinity chromatography (MCAC) was performed as

20 follows: cell lysate was diluted 1:2 with Buffer A (500 mM KCl, 20 mM Tris-Cl, pH 8.0, 10% glycerol) and applied to a 5 ml HiTrap Chelating column (Amersham Biosciences) charged with nickel. The column was washed with 10 column volumes of Buffer A, and eluted stepwise with 10 column volumes of Buffer A plus 16, 56, 167 and 500 mM imidazol, pH 8.0 at a flow rate of 1.5 ml/min and collecting 5 ml fractions. MAPKs eluted

25 in either the 167 or 500 mM imidazol fractions. After the purification described below, proteins were essentially pure as determined by silver staining of SDS-PAGE gels.

For purification of ERK, cells were grown to an OD600 of 0.7 at 37 °C and induced with 1 mM IPTG for 2 hours at 37 °C. The MCAC fraction was diluted tenfold with Buffer B (20 mM HEPES 8.0, 1 mM DTT, 1 mM EDTA, 10% glycerol) and loaded onto a 5 ml

30 HiTrap Q column (Amersham Biosciences) previously equilibrated with Buffer B plus 50 mM KCl. ERK was eluted in a 10 column gradient to Buffer B plus 500 mM KCl, at a flow rate of 1.5 ml/min, taking 2.5 ml fractions.

For purification of Jnk1, stationary phase culture was diluted to OD600 of 0.7 and grown at 37 °C for one hour, and induced with 0.5 mM IPTG for 2 hours at 37 °C. MCAC

fractions containing Jnk1 were pooled and desalted into Buffer C (20 mM HEPES 7.0, 1 mM DTT, 1 mM EDTA, 10% glycerol) plus 100 mM NaCl by passage over a PD-10 column (Amersham Biosciences). PD-10 eluate was diluted 1:5 with buffer C immediately before loading on a 1 ml HiTrap SP column (Amersham Biosciences) equilibrated in Buffer

5   C. The column was washed with 5 column volumes Buffer C, 5 column volumes Buffer C plus 50 mM NaCl, eluted with a 7 column volume gradient to Buffer C plus 300 mM NaCl, and finished with 5 column volumes of Buffer C plus 300 mM NaCl. The SP column was run at 0.5 ml/min collecting 0.5 ml fractions.

         Ion-exchange chromatograpy conditions for p38gamma, h-Ras, and RhoA were

10   identical to ERK, except KCl was substituted for NaCl in Buffer D additions. For purification of p38gamma, cultures were grown to an OD600 of 0.8 and induced with 0.5 mM IPTG for 3 hours at 30 °C. For purification of RhoA and h-Ras, cells were grown at 37 °C to an OD600 of 0.8 at 37 °C, and induced with 0.5 mM IPTG for 3 hours. MCAC fractions were diluted two fold with ddH2O, and dialyzed into Buffer D (20 mM HEPES 7.4,

15   1 mM DTT, 1 mM EDTA, 10% glycerol) plus 50 mM NaCl. Dialysate was applied to a 1 ml HiTrap Q column (Amersham Biosciences), washed with 5 column volumes Buffer D plus 50 mM NaCl, eluted with 8 column volumes to Buffer D plus 500 mM NaCl, and flushed with 3 column volumes Buffer D plus 500 mM NaCl. Column flow rate and fractionation parameters were identical to Jnk1 purification.

20   For purification of p38delta, cells were grown at 37 °C to OD600 1.0 and induced with 0.5 mM IPTG for 2 hours. MCAC fractions were dialysed against Buffer B plus 100 mM KCl, then diluted threefold with Buffer B immediately before loading on a 1 ml HiTrap SP column. The column was washed with 5 column volumes of Buffer B plus 50 mM KCl, and eluted with 10 column volumes to Buffer B plus 500 mM KCl, at a flow rate of 0.5

25   ml/min taking 0.5 ml fractions.

         For purification of Mek1, a fresh transformation was grown for 12 hours at 30 °C, split 1:100 into fresh media, and grown to OD600 0.7 at 37 °C, then induced with 1 mM IPTG for 3 hours. MCAC fractions were desalted on a PD-10 column into Buffer E (40 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol) and applied to a 1 ml HiTrap Q

30   column. The column was washed with 10 CV Buffer E and Mek1 was eluted in a single step of Buffer E plus 85 mM NaCl. Q column run parameters were as for Jnk1; fractions containing Mek1 were collected and dialyzed into Buffer F (150 mM NaCl, 10 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA) and applied to a Superdex 75 column (Amersham Biosciences). The Superdex 75 column was run at 1 ml/min and 1 ml fractions were

collected. Fractions containing Mek1 were pooled and concentrated tenfold on a Centricon YM-10 (Amicon), and glycerol was added to 10% v/v before proteins were frozen in liquid nitrogen.

For purification of Mek1-DD (Huand and Erikson, 1994), cells were grown to

5   OD600 of 0.7 at 37C and induced with 0.5 mM IPTG for 3 hours. Purification conditions were identical to RhoA and H-ras.

Example 10. Activation of ERK and generation of ppERK.

Recombinant ERK and Mek1-DD were mixed in a 20:1 molar ratio in 1X Kinase

10   Activation Buffer (100 mM NaCl, 10 mM MgCl₂, 10 mM HEPES, pH 8.0, 10% glycerol). ATP was added to 1 mM and the mixture incubated for 30 minutes at 23 °C. Excess ATP was removed from the reaction by desalting on a PD-10 column into Buffer G (20 mM HEPES, pH 8.0, 2 mM DTT, 10% glycerol) plus 25 mM NaCl. The eluate was loaded on a 1 ml HiTrap Q column and washed with 20 column volumes of Buffer G plus 100 mM '

15   NaCl. ppERK was eluted with a 60 column volume gradient to Buffer G plus 300 mM NaCl at 0.5 ml/min, collecting 0.5 ml fractions. Activated ERK eluted at 176 mM NaCl, while inactivated ERK eluted at 228 mM NaCl. Fractions containing ppERK were diluted six fold with buffer G and reapplied to a 1 ml HiTrapQ column. The column was washed with 50 mM NaCl, and the ppERK was eluted with a single step of Buffer G plus 500 mM NaCl.

20

Example 11. *In vitro* selection of cis-hammerhead derived ERK and ppERK NASMs

Library design. Figure 1B, illustrates an RNA ribozyme library derived from a hammerhead sequence pool consisting of up to 10^{17} variants of randomized sequences appended to the hammerhead ribozyme motif (Figure 19). The ribozyme library is prepared

25   on DNA synthesizer. Random nucleotides are incorporated during the synthesis to generate pools of roughly 10^{17} molecules. Figure 19 illustrates linker scanning library designed to identify cis-hammerhead NASMs that are modulated by the protein kinase ERK. The linker library was generated by appending an ERK target modulation domain to the randomized linker domain to create a library of potential ERK-modulated cis-hammerhead NASMs.

30   The linker library of ERK-modulated cis-hammerhead NASMs consists of up to 65,000 variants. Most molecules in the randomized NASM pools are non-functional NASMs.

Selection of NASMs. Sorting among the billions of NASMs to find the desired molecules starts from the complex sequence pool, whereby desired target-modulated NASMS are isolated through an iterative *in vitro* selection process: in addition to the target-

activated ribozymes that one desires, the starting pool is usually dominated by either constitutively active or completely inactive ribozymes. The selection process removes both types of contaminants. In a following amplification stage, thousands of copies of the surviving sequences are generated to enable the next round of selection. During

5   amplification, random mutations can be introduced into the copied molecules — this 'genetic noise' allows functional nucleic acid sensor molecules to continuously evolve and become even better adapted as target-activated enzymes. The entire experiment reduces the pool complexity from 10^{17} molecules down to around 100 NASMs that require detailed characterization.

10   *In vitro* Selection Method. Negative selections were set up in which 100 µl reactions containing the library (c.f. Figure 19, where the linker library of ERK NASMs consists of up to 65,000 variants, alternatively the library consists of up to 10^{17} variants of randomized sequences appended to the hammerhead ribozyme motif) of target modulated ribozymes containing randomized target modulation domains and randomized linker regions

15   (1 µM), were incubated at 72 °C, 5 seconds, followed by 25°C for 1hr in a reaction buffer containing KCl (0-150 mM), HEPES pH 7.5 (20 mM), $MgCl_2$ (20 mM), EDTA (0.5 mM). Cleaved RNA molecules (radiolabeled internally) were then removed from the mixture by electrophoresis through a 7M urea, 8% PAGE gel, followed by cutting the uncleaved band from the gel. Detection of cleaved and uncleaved RNAs were carried out using a phosphor-

20   imager system. The uncleaved hammerhead-derived RNAs were then subjected to a round of positive selection for ERK, or ppERK, target modulation, by incubating the pool of RNA in selection to which the protein target (50nM to 1 µM ERK, or 50nM to 1 µM ppERK) were added, and the mixture was further incubated at 37 °C for 30 minutes. Cleaved RNA molecules (radiolabeled internally) were then isolated and removed from the mixture by

25   electrophoresis through a 7M urea, 8% PAGE gel, followed by cutting the cleaved band from the gel. incubated with reverse transcription mix at 65 °C for 1 hour using a thermostable reverse transcriptase. The pool was subsequently separated from the reverse transcription mixture by filtration and was then amplified using PCR first with the substrate 3-specific 5'-PCR primer and the library-specific 3'-PCR primer, and secondly with the

30   regeneration 5'-PCR primer and the library-specific 3'-PCR primer in order to add the T7 promoter. Transcriptions were performed with these PCR products directly and after denaturing PAGE purification the entire process was repeated for several rounds with assays run after every round.

          Cleavage Assays

Cleavage assays were performed using radiolabeled RNA and analytical denaturing polyacrylamide gel elecrophoresis (PAGE) (gel-based assays). Assays were performed upon both the library and clonal sequences. In a representative gel-based assay transcription was performed in the presence of alpha-^{32}P-labelled UTP, and the resultant

5   transcripts were gel-purified using denaturing PAGE. Assay mixtures were then made in which 10 µl reactions containing RNA (1u µM), and protein target (1 µM) were incubated at 37 °C for between 15min and 16h in a reaction buffer containing buffer containing KCl (0-150 mM), HEPES pH 7.5 (20 mM), MgCl$_2$ (20 mM), EDTA (0.5 mM). The resultant samples are quenched by the addition of EDTA and the relative extents of cleavage

10   measured by comparison of the intensity of the corresponding bands on a denaturing PAGE observed by reading a phosphorimager plate that had been exposed to the gel.

See general Example 1A for a discussion of the cloning, sequencing and characterization of individual NASMs.

15   **Example 12. Nucleic acid sensor molecules modulated by ERK and phosphorylated ERK generated by engineering target modulation domains into hammerhead catalytic domains.**

ERK and ppERK modulated nucleic acid sensor molecules were generated by a strategy combining both engineering and *in vitro* selection, Figure 19. Target modulation

20   domains selected for binding to ERK and to the phosphorylated form of ERK (ppERK) are the starting point of the engineering efforts (Seiwert *et al.* 2000). These target modulation domains when isolated as discrete aptamers specifically recognize ERK but do not detectably interact with other mitogen-activated protein kinases such as Jun N-terminal kinase or p38 (as monitored by the ability to inhibit kinase activity). An aptamer (target

25   modulation domain) selected for ppERK binding efficiently discriminates between phosphorylated and non-phosphorylated forms of the protein, binding ppERK with a K_D of 4.7 nM and ERK with a K_D of 50 nM (Seiwert *et al.* 2000). Simply engineering an ERK aptamer sequence onto a stably duplexed linker domain appended to the hammerhead ribozyme motif as shown in Figure 21, results in non-functional NASMs, *e.g.*, certain

30   ribozyme NASMs, derived through direct engineering, when assayed as described above, exhibit little or no cleavage activity in the absence of ERK and no detectable ERK-modulation of cleavage activity in the presence of ERK. Inactive NASMs remained unresponsive to added ERK protein in concentrations ranges tested from 50 nM to 5 uM. Hence, ERK concentrations greater than 1000 fold over the aptamer K_D values does not

result in target modulation of the NASM, suggesting that functional NASM activity will require more than a functional target modulation domain appended to a ribozyme motif.

In vitro selection of cis-hammerhead NASMs based on the 65,000 member ERK target modulated ribozyme library shown in Figure 19, were performed as described in Example 1A. *In vitro* selection of hammerhead derived ERK and ppERK NASMs, and the subsequently identified and characterized a series of ERK-modulated NASMs are shown in Figure 19, and in Table 11. The detailed methods for the analysis ERK-modulated NASM clones is given in Example 1A Cloning, sequencing and characterization of individual NASMs.

Example 13. *In vitro* selection of L1-ligase derived ERK and ppERK NASMs

Starting from a random linker domain library of L1-ligase derived NASMs as shown in Figure 31, we carried out *in vitro* selection to identify ERK-modulated and or ppERK-modulated L1-ligase NASMs. Negative selections were set up in which 100 µl reactions containing the library of ribozymes (1 µM), effector oligonucleotide (1.5 µM) and substrate 3 (5 µM) were incubated at 25 °C for between 16 and 160 h in a reaction buffer containing KCl (150 mM), HEPES (20 mM), MgCl$_2$ (10 mM), EDTA (1 mM), DTT (1 mM), tRNA (0.1 mg/ml) and glycerol (10% w/v). Ligated RNA molecules were then removed from the mixture by incubation with immobilized neutravidin. The flow through was collected by filtration and to this was added more substrate 3 (2 µM), protein target (1 µM ERK or ppERK) and RNase inhibitor were added and the mixture was further incubated at 25 °C for between 15 minutes and 1 hour. Ligated RNA molecules were then captured by incubation with immobilized neutravidin and, after washing, the matrix was incubated with reverse transcription mix at 65 °C for 1 hour using a thermostable reverse transcriptase. The matrix was subsequently separated from the reverse transcription mixture by filtration and was then amplified using PCR first with the substrate 3-specific 5'-PCR primer and the library-specific 3'-PCR primer, and secondly with the regeneration 5'-PCR primer and the library-specific 3'-PCR primer in order to add the T7 promoter. Transcriptions were performed with these PCR products directly and after denaturing PAGE purification the entire process was repeated for several rounds with assays run after every round.

Ligation Assays

Ligation assays were performed using radiolabeled RNA and analytical denaturing polyacrylamide gel elecrophoresis (PAGE) (gel-based assays), or by PCR utilizing the substrate 3-specific 5'-PCR primer (PCR-based assay). Assays were performed upon both

the library and clonal sequences. In a representative gel-based assay transcription was performed in the presence of alpha-^{32}P-labelled UTP, and the resultant transcripts were gel-purified using denaturing PAGE. Assay mixtures were then made in which 10 µl reactions containing RNA (1u µM), effector oligonucleotide (1.5 µM), substrate 3 (5 µM) and

5 optionally RNase-inhibitor and protein target (1 µM) were incubated at 25 °C for between 15min and 16h in a reaction buffer containing protein target (1 µM ERK or ppERK), KCl (150 mM), HEPES (20 mM), MgCl2 (10 mM), EDTA (1 mM), DTT (1 mM), tRNA (0.1 mg/ml) and glycerol (10% w/v). The resultant samples were quenched by the addition of EDTA and the relative extents of ligation were measured by comparison of the intensity of

10 the corresponding bands on a denaturing PAGE observed by reading a phosphorimager plate that had been exposed to the gel. PCR assays were performed using the same reaction mixture except that the relative extents of ligation in different samples were compared by observing the relative rates of appearance of PCR products using the substrate 3-specific 5'-PCR primer and the library-specific 3'-PCR primers. The detailed methods for the analysis

15 ERK-modulated NASM clones is given in Example 1A, Cloning, sequencing and characterization of individual NASMs.

Example 14. Rational design and Engineering of ERK dependent ligases.

ERK-dependent ligase ribozymes were built on the catalytic core of the L1 ligase

20 ribozyme of Robertson and Ellington (2000; NAR 28, 1751-1759) in which the non-conserved, stem C element was replaced with the ERK-interacting domain and joined to the catalytic core by a 2-4 base pair helical element, or "communication module". Ten different ribozymes were designed, identical in sequence except for the bases in the linker domain (Figure 32). (SEQ ID NOs:109-116)

25 Templates for transcription by T7 RNA polymerase were prepared for each ribozyme by PCR amplification using a set of three overlapping primer oligonucleotides. The forward, or 5' primer TAATACGACTCACTATAGGACTTCGGCGAAAGCCGTTCGACC (SEQ ID NO:315), included the T7 RNA polymerase promoter and sequence corresponding to the 5'-proximal

30 region of the L1 ligase catalytic core. The other two primers corresponded to sequences spanning the 3'-proximal portion of the L1 ligase core ATTCGAGATGTCCTTGGACCAAAGCCGCACCTAACCTCCTGTCTAAG (SEQ ID NO:316) and the ERK interacting domain (including sequences), respectively. RNA was synthesized by *in vitro* transcription (Milligan & Uhlenbeck (1989) Methods Enzymol 180,

51-62) using the T7-MEGAshortscript™ transcription kit from Ambion, and purified by gel-filtration (to remove transcription buffer components) and/or polyacrylamide gel electrophoresis (PAGE). The purified RNAs were then quantified by their absorbance at 260 nm, and stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at -20 °C.

5   Radiolabeled RNAs were prepared exactly as described above, except that α-^{32}P-UTP was included in the *in vitro* transcription reaction.

Example 15. Rational design and Engineering of ppERK dependent ligases.

ppERK-dependent ligase ribozymes were built on the catalytic core of the L1 ligase
10   ribozyme of Robertson and Ellington (2000; NAR 28, 1751-1759) in which the non-conserved, stem C element was replaced with the ERK-interacting domain and joined to the catalytic core by a 2-4 base pair helical element, or "communication module". Fourteen different ribozymes were designed, identical in sequence except for the bases in the linker domain (Figure 41) (SEQ ID NO:352).

15   Templates for transcription by T7 RNA polymerase were prepared for each ribozyme by PCR amplification using a set of three overlapping primer oligonucleotides. The forward, or 5' primer
TAATACGACTCACTATAGGACTTCGGCGAAAGCCGTTCGACC (SEQ ID NO:315), included the T7 RNA polymerase promoter and sequence corresponding to the 5'-proximal
20   region of the L1 ligase catalytic core. The other two primers corresponded to sequences spanning the 3'-proximal portion of the L1 ligase core
ATTCGAGATGTCCTTGGACCAAAGCCGCACCTAACCTCCTGTCTAAG (SEQ ID NO:316) and the ppERK target modulation domain (including sequences), respectively. RNA was synthesized by *in vitro* transcription (Milligan & Uhlenbeck (1989) Methods
25   Enzymol 180, 51-62) using the T7-MEGAshortscript™ transcription kit from Ambion, and purified by gel-filtration (to remove transcription buffer components) and/or polyacrylamide gel electrophoresis (PAGE). The purified RNAs were then quantified by their absorbance at 260 nm, and stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at -20 °C. Radiolabeled RNAs were prepared exactly as described above, except that α-^{32}P-UTP was
30   included in the *in vitro* transcription reaction.

Example 16. Linker-region selection of effector-dependent ligases

Oligonucleotides

Synthetic DNA and the DNA-RNA chimeric substrates were synthesized using standard solid-phase methodology and purified by denaturing (8M urea) polyacrylamide gel electrophoresis (PAGE). Random-sequence regions of synthetic DNA were generated using a single mixed "N" bottle on the DNA synthesizer that contains all four nucleotide

5   phosphoramidites mixed in proportions. Variable-length regions of synthetic DNA were generated by interrupting the synthesis and removing part of the reaction mixture, re-starting the synthesis and then replacing the removed material after a subsequent interruption. Templates for transcription were generated by PCR, and the transcripts thus generated were also purified by denaturing (8M urea) polyacrylamide gel electrophoresis

10   (PAGE). Gel-purification is followed by the localization of nucleic acids within the gel by UV-shadowing, nucleic-acid containing gel pieces are excised and the purified nucleic acids are recovered by electroelution. The following synthetic oligonucleotides were utilized:

Oligonucleotides common to all selections:

Substrate 3 (DNA, RNA underlined)

15   Biotin-TEG-CATGCGACCTTACGATCAGATGACCTUGCACU (SEQ ID NO:321)

Substrate 3-specific 5'-PCR primer

CATGCGACCTTACGATCAGAT (SEQ ID NO:322)

20

Regeneration 5'-PCR primer

TCTAATACGACTCACTATAGGACTTCGGCGAAAGC (SEQ ID NO:323)

ERK selection-specific oligonucleotides:

25   Library (DNA) (random regions 3-5 nucleotides in length)

GGACTTCGGCGAAAGCCGTTCGACCNNN(N)(N)AAGGAGGATTTCCGAAAGCGG

CTACGGTCCGCCNNN(N)(N)CTTAGACAGGAGGTTAGGTGCGTAGGTAACCGAT

AGTTCCG (SEQ ID NO:324)

30   Effector (2'-O-methyl DNA), 3'-PCR primer (DNA) and RT primer (DNA)

CGGAACTATCGGTTACCTAC (SEQ ID NO:325)

ppERK selection-specific oligonucleotides:

Library

GGACTTCGGCGAAAGCCGTTCGACCNNNNN(N)(N)CAGACGCTAGCGAATTGGTT
CCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGNNNNN(N)(N)CTTAGACA
GGAGGTTAGGTGCGTCAATGCTGCAAGTTACTG (SEQ ID NO:326)

5   Effector (2'-O-methyl DNA), 3'-PCR primer (DNA) and RT primer (DNA)
       CAGTAACTTGCAGCATTGAC (SEQ ID NO:327)

       bFGF selection-specific oligonucleotides:

10   Library (random regions 5-7 nucleotides in length)
           GGACTTCGGCGAAAGCCGTTCGACCNNNNNN(N)(N)GCAACGCTACAGACA
       AGTGCANNNNN(N)(N)CTTAGACAGGAGGTTAGGTGCCCGAGTTGTTCGAACGA
       GAC (SEQ ID NO:328)

15   Effector (2'-O-methyl DNA), 3'-PCR primer (DNA) and RT primer (DNA)
       GTCTCGTTCGAACAACTCGG (SEQ ID NO:329)

       Thrombin selection-specific oligonucleotides:
       Library
20   GGACTTCGGCGAAAGCCGTTCGACCNNNNN(N)(N)ATCGAAGTTAGTAGGNNN(N)
       (N)CTTAGACAGGAGGTTAGGTGCGTCAATCGATTGCAGATCCG (SEQ ID
       NO:330)

       Effector (2'-O-methyl DNA), 3'-PCR primer (DNA) and RT primer (DNA)
25   CGGATCTGCAATCGATTGAC (SEQ ID NO:331)

Example 17. Nucleic acid sensor molecules modulated by native ERK enzyme.
       Hammerhead-derived nucleic acid sensor molecules (88 nucleotides in length) were
selected from populations of nucleic acid molecules with randomized linker domain in stem
30   II, as shown in Figure 19. The Table in Figure 19 depicts illustrative linker-domain
sequences of several of the ERK-modulated nucleic acid sensor molecules isolated from *in
vitro* selection linker randomized clones (described in detail in Example 11). Each NASM
displays a varying degree of modulation driven by addition of equal amounts (1 uM) native
ERK. Individual ERK-modulated cis-hammerhead nucleic acid sensor molecules are shown

in Figure 19. Clones 1-14, 1-13, 1-2, 1-6, 2-7, 2-2, 2-3, 2-13, 2-14, and 2-20 were tested in target modulation assays as described previously. The NASM's relative dependence on ERK is denoted in the Table in Figure 19 by the extent of activity of the ribozyme in the presence of ERK protein.

5   The time course of signal generation in the presence of nonphosphorylated ERK, phosphorylated ERK, and in the absence of protein is determined by measuring signal released over time by a radiolabeled nucleic acid sensor molecule. Significant amounts signal, corresponding to cleavage of the nucleic acid sensor molecule is observed over time only with the nonphosphorylated ERK. Clones1-2, 1-13 and 1-14 all display sensitivity to

10   ERK concentration, as shown in Figures 20 A, B and C. However, of these three clones, clone 1-14 displays the greatest enhancement in activity upon addition of ERK. Clone 1-14 is able to differentiate between varying concentrations of ERK, as indicated by the dose-dependent change in the activity of clone-14 upon the addition of 5, 10, 20, 50 and 100 nM ERK (Figure 21).

15

Example 18. ERK-modulated NASM-based competitive inhibition assays, & target-protein specific profiling biosensors.

The specificity of the interaction between clone 1-14 and ERK was assessed by measuring the activity of clone 1-14 in the presence of 50 nM ERK and increasing

20   concentrations of known protein kinase inhibitors. The kinase inhibitors staurosporine and 5-iodo-tubercidin are compounds that are known bind to in an ATP-substrate competitive manner to ERK and to modulate ERK kinase catalytic activity, decreasing its ability to phosphorylate other proteins. We tested whether kinase inhibitors would compete directly for the ATP-binding site in ERK, and thereby block NASM activation by ERK. As shown

25   in Figure 22A, increasing concentrations of the compounds staurosporin and or ITU disrupt the interaction between clone 1-14 and ERK protein, suppressing the activation of the nucleic acid sensor molecule. We have used NASM-based assay to confirm the known kinase-inhibitory IC50 values of staurosporin and ITU (5 and 1 uM, respectively). This result indicates that clone 1-14 recognizes free unliganded ERK and is not modulated by the

30   ERK-stuaroporine or ERK-ITU complex. Since, staurosporin or ITU (ATP analogs) are general kinase inhibitors, and bind to the active/ATP binding sites of kinases, these data suggest that the NASMs either bind directly to the kinase active site, or are exquisitely sensitive to the conformational state of the enzyme. Hence, when saturating levels of the kinase inhibitors are present (10 uM) in the NASM-assay, along with uM levels of ERK, the

nucleic acid sensor molecule does not signal the presence of ERK protein. Thus, the ERK-modulated NASMs are used as general HTS screening reagents for the discovery of small molecule drug leads.

Similar results to those described above for the hammerhead-derived NASMs of

5   Figure 19 have been obtained with ERK-modulated L1-ligase NASMs described in detail in Example 13. Figure 58, shows additional staurosporine competitive NASM assay results using an L1-ligase-derived NASM which is also modulated by ERK protein. Additional modifications of the 3-piece L1-ligase NASM (c.f., Figure 36, constructs 27 and constructs 28) render these biosensors suitable for cellular assays as intracellular biosensors (c.f.,

10   Figure 37 and the detailed description of the 3-piece, and 1-piece ligases described in detail in Example 22). Thus, ERK-modulated nucleic acid sensor molecules are used for cell-based drug discovery and drug candidate screening.

The data in Figures 22B and Figures 57 A and 57B indicates that ERK NASMs (clone 1-14 and constructs 27 and 28) are modulated specifically by ERK only in its fully

15   active, and not by ppERK, or by related homologues and MAP Kinase pathway molecules, For example clone 1-14 recognizes only ERK, and not related kinase MEK. Hence, NASMs and more specifically, the ERK and ppERK NASMs, are shown here to be useful specific protein profiling biosensors.

A compilation of novel hammerhead ERK-modulated nucleic acid sensor molecule

20   sequences are disclosed in Table 11.

Table 11. Cis-hammerhead nucleic acid sensor molecules

Sequences of 157 ERK activated cis-hammerhead clones.

25   Sequence
        SEQ ID

NO:

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30   140

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141

GGGCGACCCUGAUGAGGGUUGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUGGCCAAACAGNGAAANNNGNANGUGGNC

        142

35   GGGCGACCCUGAUGAGUACGGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUAGAACGAAACGGUGAAAGCCGUAGGUUGCC
143

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144

GGGCGACCCUGAUGAGUUGCGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUAUAACGAAACGGUGAAAGCCGUAGGUUGCC

40   145

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        147

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·148

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GGGCGACCUUGAUGAAGGNCAGNUAAGGAGGAUUUCCGAAAGCGGNUACGGUCAGCCAGUGGCACGAAACGGUGAAAGCCGUAGGUUGCC
281
GGGCGACCCUGAUGAGCAUGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUUAGUCGAAACGGUGAAAGCCGUAGGUUGCC
292
GGGCGACCCUGAUGANNGCUGNUAGGAGGAUUUCCGAAANUGCUUCUGGCCCGCANUUANNCCGAACGGGCNAAANCGAANGGUGNC
283
GGGCGACCCUGAUGAGUAGGCUAAGGAGGAUUUCGAAAGCGGCUACGGUCCGCCAGUGUGCCGAA
284
GGGCGACCCUGCUGAGCNACGCUANGAGGAUUUCCGAAAGCGGCUACGGNCCGCCAGUCNGACGAAACGGUGAAAGCCGUAGGUUGCC
285
Construct 1-14
GGGCGACCCUGAUGAGGGUCGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGACGUCGAAACGGUGAAAGCCGUAGGUUGCC
286
Construct 1-13
GGGCGACCCUGAUGAGUCUUCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUACGUCGAAACGGUGAAAGCCGUAGGUUGCC
287
Construct 1-2
GGGCGACCCUGAUGAGCCUUCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGACGUCGAAACGGUGAAAGCCGUAGGUUGCC
288
Construct 1-6
GGGCGACCCUGAUGAGUCAUGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGACGUCGAAACGGUGAAAGCCGUAGGUUGCC
289
Construct 2-7
GGGCGACCCUGAUGAGCCUUGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUCAGUCGAAACGGUGAAAGCCGUAGGUUGCC
290
Construct 2-2
GGGCGACCCUGAUGAGCCUCGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGCUAGCGAAACGGUGAAAGCCGUAGGUUGCC
291
Construct 2-3
GGGCGACCCUGAUGAGAACUGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUUUAACGAAACGGUGAAAGCCGUAGGUUGCC
292
Construct 2-13
GGGCGACCCUGAUGAGUAAAGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUCUGGCGAAACGGUGAAAGCCGUAGGUUGCC
293
Construct 2-14
GGGCGACCCUGAUGAGUUUUGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUGAUCCGAAACGGUGAAAGCCGUAGGUUGCC
294
Construct 2-20
GGGCGACCCUGAUGAGGCGAGCUAAGGAGGAUUUCCGAAAGCGGCACGGUCCGCCAGUUUAACGAAACGGUGAAAGCCGUAGGUUGCC
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These results demonstrate that the nucleic acid sensor molecule is specific for the non-phosphorylated form of the ERK.

Example 19. Cis-hammerhead-derived nucleic acid sensor molecules activated by
5 **ppERK**

A stem-selection library of hammerhead-derived nucleic acid sensor molecules modulated by ppERK is shown in Figure 23A. ppERK-modulated nucleic acid sensor molecules were designed to by joining ppERK a target modulation domain (TMD) to a hammerhead core sequence via a linker region. Figure 23A shows the general structure of

10 ppERK-modulated hammerhead ribozyme with the linker region depicted as a randomized sequence (N6). Individual ppERK dependent *cis* hammerhead RNA sequences are shown in Figure 23B.

Figure 24 is a table displaying different properties of the *cis* hammerhead ERK sensors shown in Figure 23B. Each construct displays variability in its sequence, its activity

15 and its stability.

Figure 25 shows data for the ppERK-NASMs that have been tested in target modulation assays using in situ generated ppERK (using constitutively activated MEK activate to generate ppERK from native ERK). ppERK has been generated *in situ* by direct phosphorylation of ERK with constitutively active MEK mutant, MEK-EE in the presence

20 of $MgCl_2$ and ATP. Figure 25 also shows data for radiolabeled nucleic acid sensor molecules for ppERK-hammerhead NASM constructs 6, 10, and that were incubated at 37° C for 30 minutes in the absence of protein; in the presence of 2 μM ERK, 0.14 μM constitutively active MEK (MEK-EE); in the presence of 2 μM ERK, 0.14 μM constitutively active MEK-EE, 1 mM ATP; in the presence of 1 mM ATP only. All these

25 reactions were performed in the following buffer of 20 mM HEPES (pH 7.5), 10 mM MgCl2, and 0.1 mM EDTA. The mixtures of ERK, ERK + MEK-EE, and ERK+MEK+EE with ATP were incubated in the presence of 10mM MgCl2 prior to mixing with radiolabeled nucleic acid sensor molecule. After the incubation, the nucleic acid sensor molecule reaction mixtures were subjected to 5% acrylamide gel containing 8 M Urea as

30 shown in panel B of Figure 25., in order to examine the NASM endonuclease activity. Three constructs displayed an increase in endonuclease activity in the presence of ERK + MEK + EE with ATP as shown in Figure 25. These data indicate general methods for a NASM-based ERK activation assay: as shown in Figure 25, NASMs are used to directly

detect MEK conversion of ERK to ppERK as detected by ppERK-modulation of nucleic acid sensor molecules.

Example 20. Characterization of the ERK and ppERK modulated L1-Ligase derived
5   **NASMs**

A template for an ERK dependent ligase is shown in Figure 31. Figure 32 discloses the different clones of ERK dependent ligases based on this template. The ERK dependence of these clones is shown in Figure 33. Constructs 17 and 19 are particularly sensitive to 2μM ERK. Construct 19, also known as Clone E, was tested to see if it could
10   be used to detect increasing concentrations of ERK. Construct 19 was found to have a distinct signal at 1 μM, 100 nM, and 10nM ERK. Time dependency was also tested for Construct 19 as shown in Figure 34. Increasing signal from Construct 19 could be seen at 1, 2, 3, and 4 hours. Concentration dependence of Construct 19 (Clone E) is also shown in the graph in Figure 35. Construct 19 can differentiate between different concentrations of
15   ERK. Two formats that can be used with nucleic acid sensor molecule ligases are the 3-piece ligase platform, and the 1-piece ligase platform, both shown in Figure 36. The different platforms allow the nucleic acid sensor molecules to be used in different applications. For example, the 3-piece could be used *in vitro*, while the 1-piece could be used *in vivo*, even intracellularly. The performance of these two formats is shown in Figure
20   37. There is also a 2-piece platform shown generally in Figure 38 and shown as ERK regulated 2-piece ligases as shown in Figure 39A. The 2-piece platform of Construct 19 is also ERK dependent as shown in Figure 39B.

Ligase nucleic acid sensor molecules can be used in the presence of cell extracts. Construct 19 shows ERK activation dependence in the presence of 50% HeLa cell extract as
25   shown in Figure 40.

Ligase based nucleic acid sensor molecules were also found that were specifically activated by ppERK. Figure 41 shows a template for the ppERK sensitive ligase. Figure 42 show the specific sequences left generalized in Figure 41. ppERK sensitivity was shown in the clones described Figure 57 shows activation of 3-piece Construct 19 (DG.20.58E)
30   (ERK2-dependent) (SEQ ID NO:111) and TK.16.118.M (ppERK2-dependent) (SEQ ID NO:123) ligases in the presence of purified proteins. Ligation assays (10 mM MgCl₂, 100 mM NaCl, 30 mM Tris, pH 7.5) were performed at room temperature for 1 hour, using 1 μM ligase RNA, 1 μM effector oligonucleotide, 2 μM substrate oligonucleotide and 1 μM

protein. RNAs were incubated in the presence of: (A) purified ERK, in either its non-phosphorylated or diphosphorylated form; or (B) other members of the MAP kinase pathway (Ras, MEK1 and p38), and ricin. Ligase activity was measured in solution, using real-time RT-PCR detection.

5   It has been shown that Construct 19 still has activity in low magnesium ion. In one embodiment of the invention, these nucleic acid sensor molecules can be used in cell extracts and intracellularly, to detect ppERK. There is very low magnesium ion concentrations in these environments, so it is important that these nucleic acid sensor molecules maintain function even in low concentrations of magnesium ion.

10   Figure 42 shows the full sequences of the different clones of ppERK sensitive nucleic acid sensor molecules in the 3-piece format.

Example 21. Amplicon Detection

Amplicon dependent nucleic amplification is a high-throughput method for cell or
15   tissue profiling of proteins and metabolites. The method can include using real-time PCR to follow the activity of nucleic acid sensor molecules over time to follow the changing concentrations of a protein or metabolite.

The method can also comprise the use of multiplexed chips. A chip can be exposed to a sample solution containing proteins or metabolites to be analyzed. Using multiple
20   nucleic acid sensor molecules on one chip, the concentrations of many analytes can be shown simultaneously. This can also be extended to cellular assays as shown in Figure 72.

TaqMan can also be used in Protein-PCR, as shown in Figure 58. Figure 58 shows solution-based detection of ligase activity using real-time RT-PCR. Ligation reactions (10 mM $MgCl_2$, 100 mM NaCl, 30 mM Tris, pH 7.5) were performed for 0.5-2 hours at room
25   temperature using 1 μM 3-piece construct 19 (DG.20.58E), (SEQ ID NO:111) ligase, 2 μM effector oligonucleotide, 2 μM substrate oligonucleotide and purified ERK protein. Part A shows the effect of 293 cell extracts on the ERK concentration dependence of ligase activity. The increase in ligation product observed with increasing concentrations of ERK (0-3.2 μM) is shown in the presence of buffer only (circles) or buffer containing 10% 293
30   cell extract (squares). (inset) The ERK2 concentration dependence of ligation is shown as the decrease in cycles of amplification required for detection of ligase product RNA, as measured by real-time RT-PCR. In Panel B ligation in the presence of 1 μM ERK and increasing concentrations of the kinase inhibitor staurosporine is shown. ERK nucleic acid

sensor molecules can be used to detect ERK in a concentration dependent manner even in the presence of 293 cell extract. This detection is sensitive to the presence of staurosporine, a kinase inhibitor, which indicates that the activity of ERK is important to the detection of ERK by the nucleic acid sensor molecule.

5   Amplicon dependent nucleic acid amplification technique can be used to show the downstream effects of EGF and MEK inhibitor administration in a cell. The diphosphorylated ERK is detected in the presence of EGF and not in the presence of the MEK inhibitor as would be expected. This technique can be used to screen other substances for their ability to have ERK phosphorylated. In one embodiment, this technique could be

10   used to screen for carcinogens in certain cell types.

Example 22. Analysis of radiolabeled ligation products in 3, 2, 1-piece systems.

The ERK-modulated 3-piece ligase is converted to the 1-piece ligase NASM by displacement of the effector oligonucleotide binding domain by a stable tetraloop , as

15   depicted in Figure 36. The deletion of the effector oligonucleotide binding domain of construct 27 (in general), and construct 27, specifically, to generate construct 28 has no effect on the switch factor of the 1-piece ligase NASM (in comparison to the 3-piece ligase NASM), nor does it have an effect on the ERK-modulation KD value for the 1-piece ligase NASM (in comparison to the 3-piece ligase NASM), Figure 37. The exact sequence

20   constraints for the displacement of the effector oligonucleotide binding domain in construct 27 to generate construct 28, were in fact delineated by the analysis obtained from the lysozyme-modulated 1-piece ligase NASMs shown in Figure 26. The data in Figure 26 also indicates that the length of the stem region proximal to the tetraloop has a significant effect on ligase (e.g. self-circularization) efficiency. The self-circularizing ligase NASMs are then

25   used as biosensors in intracellular target modulation assays, Figure 30. Detailed assay conditions are described in subsequent Examples.

Additionally, the ERK-modulated 3-piece ligase is converted to the 2-piece ligase NASM by linking the effector oligonucleotide substrate to the oligonucleotide substrate (Figure 38), effectively creating the oligonucleotide supersubstrate, which base pairs with

30   both the effector oligonucleotide binding domain and the oligonucleotide substrate binding domain. The 2-piece ligase NASM configuration has particular utility in chip applications, described in detail in subsequent Examples (cf., Figures 75 and 79).

Ligation reactions containing ^{32}P-labeled ligase RNA were analyzed by 8-12% PAGE to separate unligated and ligated products, and quantified using a PhosphorImager

(Molecular Dynamics). Initial rates of ligation were calculated by linear regression from plots of the increase in ligation product over time.

Analysis of ligation products can also be performed by amplicon dependent nucleic acid amplification (ADNA). Amplicon dependent nucleic acid amplification (ADNA) was

5   performed using the TaqMan® One-Step RT-PCR protocol from the same Applied Biosystems. In a typical assay, approximately 0.7 nM ligase RNA (diluted from the EDTA-quenched ligation reaction) was mixed in 25 μL of TaqMan® One-Step master mix with 300 nM each of both the forward (5'-GCGACCTTACGATCAGATGAC (SEQ ID NO:317)) and reverse (5'-CCGCACCTAACCTCCTGTCTAA (SEQ ID NO:318)) PCR

10   primers (Integrated DNA Technologies), and 350 nM TaqMan® fluorescent probe oligonucleotide (ERK-ligase: 5'-6FAM-AAGGAGGATTTCCGAAAGCGGCTACG-TAMRA (SEQ ID NO:319); ppERK-ligase: 5'-6FAM-CGCTAGCGAATTGGTTCCTCGAAAGG-TAMRA (SEQ IDNO:320)). RT-PCR amplification was performed in the ABI Prism™ 7000 Sequence Detection System by

15   incubating the reaction mixes at 48 °C for 30 min to generate the cDNA complement of the ligated RNA product, followed by 40 cycles of PCR amplification. The increase in fluorescence upon amplification is quantified in units of C_T (threshold cycles), equal to the number of cycles of amplification required for sample fluorescence to exceed background levels. The molar quantity of ligation product can be calculated from these data by

20   comparison with a standard curve generated using a series of known product (ligated biosensor) concentrations.

Example. 23 Measurement of affinity between ERK protein and ERK-dependent nucleic acid sensor molecules.

25   The affinity of the ERK protein target for the ERK-dependent nucleic acid sensor molecule can be measured directly, by a filter-binding assay, or indirectly, by an activity-based assay. In the direct method, increasing concentrations of ERK protein target (0-10 μM) are equilibrated with trace (<1 nM) concentrations of ^{32}P-labeled, ERK-dependent nucleic acid sensor. The samples are drawn through a sandwich of Protran BA85

30   nitrocellulose (Schleicher and Schuell) and Hybond P nylon (Amersham-Pharmacia) filter papers, using a 96-well vacuum manifold. Protein and protein-bound RNA species adhere to the nitrocellulose, and free RNA to the nylon. The concentration of ERK protein/sensor complex is plotted as the fraction of the total radioactivity per sample bound to the

nitrocellulose versus total ERK protein concentration. The dissociation constant (K_D; Figure 59) is determined as the ERK protein concentration at which 50% of the nucleic acid sensor RNA is bound to protein.

In the indirect method, the activity of the ERK-dependent nucleic acid sensor molecule is measured as a function of increasing ERK protein concentration. In a typical measurement, 50 nM nucleic acid biosensor is incubated with ERK protein (0-10 µM) for one hour at room temperature. The extent of ligation at each concentration of ERK protein is determined by real-time RT-PCR (Figure 60, Panels C and D). The ligation product (nM) is plotted against ERK protein concentration, and the apparent dissociation constant (K_{act}) is determined as the protein concentration at which the extent of ligation is half-maximal. Panels A and B show ERK-dependent ligation is determined by RT-PCR on an agarose gel containing ethidium bromide.

Example 24. High throughput screening (HTS) assays using ppERK nucleic acid sensor molecules

The competitive inhibitor nucleic acid sensor molecule can be used in a HTS assay. A nucleic acid sensor molecule specific for phosphorylated ERK is used as a competitive inhibitor for ATP binding (Seiwert, Stines Nahreini et al. 2000). A competitive assay for compounds is established by incubating ERK with 10 nM nucleic acid sensor molecule in the presence of various concentrations of the inhibitors in 10 mM Tris buffer pH 7.5 containing 0.5 µg/µl tRNA and 10 mM $MgCl_2$. The reactions are terminated by addition of EDTA and the amount of reacted nucleic acid sensor molecule has been determined.

In other embodiment, the nucleic acid sensor molecules that are sensitive for the phosphorylation state of peptides (or protein substrates) can be used in HTS assays for kinase activity. The nucleic acid sensor molecules can be generated to be specific for the phosphorylation state of substrates or its peptides. HTS assay can be performed using these substrate (Mansour, Candia et al. 1996). For example, MEK can be expressed as GST-tagged protein and purified by a standard method. The activity are measured at 30°C under standard reaction conditions of 20 mM HEPES (pH 7.4), 2 mM dithiothreitol, 0.01% Triton X-100, 10 mM $MgCl_2$, 0.1 mM ATP, and 1M His-tagged ERK, at concentrations of MEK, 0.5 nM, in the presence of various concentration of compounds. After incubation, the reaction is terminated by absorbing MEK with GST-column. Phosphorylation of ERK is

quantified by nucleic acid sensor molecule. Alternatively, the nucleic acid sensor molecule can be used in western-blotting format (Bianchini, Radrizzani et al. 2001).

The nucleic acid sensor molecule specific for phosphoERK (ppERK) described above binds to ppERK, presumably in a competitive manner at the active site of the kinase

5   enzyme, and inhibits the phosphorylation of the ppERK substrate by ppERK enzyme. Up to 200 nM nucleic acid sensor molecule inhibits up to 80% of the ppERK phosphorylation of ERK substrate.

Example 25. *In vivo* assays using phosphorylation state-sensitive nucleic acid sensor

10   molecules

The phosphorylation state sensitive ppERK nucleic acid sensor molecule is used to determine drug efficacy *in vivo* (*e.g.*, tissue and cell culture). For example, T84 cells on glass coverslips are incubated in the presence and the absence of the MEK kinase inhibitor, and cells are fixed by 4% paraformaldehyde and permeabilized using 0.3% Triton X-100.

15   The slides are incubated with FRET nucleic acid sensor molecule (Bianchini, Radrizzani et al. 2001). The localization of phosphorylated substrate can be observed using a confocal microscope.

Alternatively, phosphorylation-state-sensitive nucleic acid sensor molecule are incorporated into reporter-gene constructs. These constructs are introduced into cells, and

20   phosphorylation of the substrates is monitored.

Nucleic acid sensor molecules made of nuclease resistant forms of hammerhead, - ligase or -hairpin ribozymes are transfected into mammalian cells using standard lipofectamine reagents or liposomal solutions known to effect internalization an cellular uptake of polynucleotides. If desired, the nucleic acid sensor molecules are attached to

25   polypeptides such as tat or antennapoedia or the like and are transported into mammalian cells by methods known in the art.

The activity of nucleic acid sensor molecule systems is followed by changes in fluorescence if the nucleic acid sensor molecule is fluorescently tagged, *i.e.*, is an optical nucleic acid sensor molecule or by changes in size as determined by RT-PCR.

Example 26. Detection of analytes in crude biological samples.

Nucleic acid sensor molecules can also be used to detect the presence of and measure the concentration of non-nucleic acid analytes in crude biological samples as

5   shown in Figure 56.

As noted above, nucleic acid sensor molecules can be created whose activity is activated in the presence of a specific target molecule. These nucleic acid sensor molecules together with any required substrates, can be combined with a complex mixture of biological origin and allowed to undergo reaction. Subsequent quantification of the product

10   of the reaction can be used to infer the concentration of the analyte in the sample.

In a preferred embodiment, the crude biological sample is human serum or mammalian cell extract. The nucleic acid sensor molecule catalyzes self-ligation with an oligonucleotide substrate. RNAse-inhibitors such as ribonucleoside vanadyl complex can be used to prevent degradation of the product. The product of catalysis can be measured by

15   a variety of means (e.g., a gel mobility shift assay as shown in Figure 50).

Ribonucleoside vanadyl complex (RVC) can be used as a general purpose inhibitor of endogenous RNAses to enable the use of allosteric ribozymes in assays of samples containing RNAse activity (e.g. human serum, mammalian cell extracts, bacterial extracts). RVC inhibits RNA degradation mediated by generic RNAses as well as RNAse H-like

20   activities that specifically target RNA-DNA hybrids. Inhibition of RNAse activity prevents degradation of the reaction product generated by ribozyme action that ultimately forms the basis for readout in a ribozyme-based assay. In a specific example, a lysozyme-dependent nucleic acid sensor molecule RNA with the sequence 5'GGACCUCGGCGAAAGCUAACGUCUCAUGGCUAAAUUGCCAUGUUGCUACAA

25   AUGAUAUGACUAGAGAGGUUAGGUGCCUCGUGAUGUCCAGUCGC (SEQ ID NO:312) is prepared through transcription off a double-stranded DNA template using T7 polymerase (Ambion MegaShortscript kit, Ambion, Austin, TX), including trace amounts of [alpha-^{32}P]UTP. For the ligation reaction, 1 μM nucleic acid sensor molecule RNA, 2 μM DNA effector oligo 5' GCGACTGGACATCACGAG (SEQ ID NO:313) and 2 μM

30   oligonucleotide substrate 5' GTACGATGCGATGCTAGCGATTGTTgugcacu (SEQ ID NO:314) (DNA: upper case; RNA: lower case) were incubated in the presence or absence of 1 μM hen egg white lysozyme in reaction buffer (50mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl$_2$) as previously described (Robertson MP & Ellington AD, Nature Biotechnol. 2001, 19, 650-655). HeLa extract is added to some of the samples (10% v/v), plus one of various

RNase inhibitors (human placenta RNase inhibitor (Sigma, St. Louis, MO), RNasin (Ambion, Austin, TX) and vanadyl ribonucleoside complex, RVC, (Sigma, St. Louis, MO)). After 60 min at room temperature, the reaction is stopped by addition of loading buffer containing formamide, and the samples are analyzed by electrophoresis on a 6% denaturing

5   gel, followed by Phosphorimager scanning (Figure 51, left). The HeLa extract samples that contain protein-based RNase inhibitors show significant broadening of the precursor RNA band compared to a buffer-only control, suggesting RNase H degradation where the RNA is bound to the DNA effector oligonucleotide. The sample containing the VRC RNase inhibitor does not show any significant degradation. All of the samples show lysozyme-

10   dependent product formation. To further test this hypothesis, additional test are done with an analog of the nucleic acid sensor molecules where the oligonucleotide effector domain is deleted

(5' GGACCUCGGCGAAAGCUAACGUCUCAUGGCUAAAUUGCCAUGUUGCUACA AAUGAUAUGACUAGAGAGGUUAGGUGC) (SEQ ID NO:332) under otherwise

15   identical conditions (Figure 51, right). No broadening of the RNA bands is observed, suggesting that the RNase activity was indeed specific to the RNA/DNA hybrid region. In a series of related experiments the above described reactions with the lysozyme-dependent nucleic acid sensor molecule are done in buffer to which 10% v/v human serum is added (Figure 53). Without the addition of RVC complete degradation of the RNA is observed. In

20   the presence of VRC, however, no difference in RNA stability and lysozyme-dependent product formation is observed.

By inactivating endogenous RNAse H-like activity, it is possible to carry out allosteric ribozyme assays in which the ribozymes have been immobilized on a chip surface through hybridization with a tethered DNA probe.

25

Example 27. Formatting an ERK Nucleic acid sensor molecule chip

Immobilization of nucleic acid sensor molecules on a substrate provides a straightforward mechanism for carrying out multiple arrays in parallel. Initially, the optimal attachment chemistries were determined to be used to immobilize these molecules on a solid

30   substrate. These molecules are were further configured such that their activity and allosteric behavior is maintained following immobilization. Generally, the chip is configured such that it may be placed at the bottom of a sample holder and overlaid with sample solution, target (ERK and ppERK) (and substrate oligonucleotide). Following an incubation to allow

ERK and pERK present within the sample to activate catalysis, the sample is washed away and the extent of ribozyme catalysis quantified.

Detection. Immobilized nucleic acid sensor molecules for ERK and pERK are prepared as described in the following section and are assayed for activity by monitoring either retention of end-labeled oligonucleotide substrate (for L1 ligase-based ribozymes) or release of end-labeled ribozyme (for endonucleases as originally described by (Seetharaman et al. 2001). Radioactive tracers are used for labeling RNAs and substrates. To the extent that different attachment chemistries do not interfere, both fluorescent and biotin labels are used in end-labeling the oligo substrate.

Attachment chemistries. One advantage of using nucleic acid sensor molecule arrays as opposed to protein arrays is the relative ease with which nucleic acid sensor molecules can be attached to chip surfaces. Several different chemistries for attaching nucleic acid sensor molecules to solid supports were tested and optimized, such as:

1. Conventional DNA arrays using aldehyde coated slides and 5'-amino modified oligonucleotides. The attached oligonucleotide serves as a capture tag that specifically hybridizes to a 3'-end extension on the ribozyme.

2. Nucleic acid sensor molecule RNA treated with periodate to specifically introduce an aldehyde modification at the 3'-end. Modified RNA can be used in two different ways:

- Subsequent reaction with biotin hydrazide enables RNA capture on commercially-available streptavidin coated slides.

- Subsequent reaction with adipic acid dihydrazide enables RNA capture on commercially-available aldehyde coated slides.

3. (Endonuclease) Nucleic acid sensor molecules are generated by transcription in the presence of γ-thio-GTP (introducing a unique thiol at their 5'-end) and subsequently attached to a thiol-reactive surface (*e.g.* gold-coated polystyrene as described by Seetharaman et al.).

Attachment methodologies are evaluated on the basis of the following criteria:

- efficiency – what is the yield of nucleic acid sensor molecule capture?

- capacity – what is the maximum concentration of nucleic acid sensor molecules that can be localized in a given spot size?

•   **stability** – are ribozymes efficiently retained under a variety of solution conditions and during long-term storage?

•   **detection** – do immobilization chemistries interfere with the ability to generate a detectable signal?

Reconfiguring ribozymes for activity in solid phase applications.   To the extent, that allosteric activity for immobilized ERK and pERK nucleic acid sensor molecules is diminished, three different strategies are possible:

1.   **Immobilization chemistries.** A variety of different immobilization chemistries are compared on the basis of their ability to maintain allosteric behavior. To the extent that they leave different surfaces available for protein effectors to interact with, that they tether different ends of the nucleic acid sensor molecules, and that they position the NASM either directly at the surface or displaced from the surface (in the case of streptavidin capture), different behaviors are observed depending upon the immobilization method. Protein-target activated NASMs have been shown to function in both direct and indirect attachment scenarios.

2.   **Blocking chemistries.** Blocking agents (*e.g.*, carrier proteins) are tested to determine whether losses in allosteric responsiveness are due to non-specific interactions between the allosteric activators and the chip surface.

3.   **Tethers.** Steric effects may cause decreased catalytic activity upon direct end attachment to a solid support. Arbitrary sequence tethers are added as needed to increase the spacing between the attachment end and the core of the ribozyme.

While endogenous activators and nucleases do not appear to be an issue for the lysozyme-responsive nucleic acid sensor molecules, the following strategies are used in the eventuality that they are a problem for the ERK/pERK (or other) nucleic acid sensor molecules.

Nucleic acid sensor molecule pre-screening. As noted above, individual clones isolated from the selection experiment are tested early for allosteric activation in the presence of ERK-depleted extracts. Molecules that respond to endogenous non-specific activators are eliminated from further consideration.

Negative selection with depleted extracts. To the extent that all isolated nucleic acid sensor molecules are activated by ERK-depleted extracts, depleted extracts are included in the negative selection step of the selection process.

Nuclease inhibition. Commercially available RNase inhibitors and competing

5   RNAse substrates (*e.g.* tRNA) are added to test samples.

Kinetic considerations. RNAse-mediated degradation of the nucleic acid sensor molecule proceeds at a rate in competition with the rate of nucleic acid sensor molecule catalysis. As such, nucleic acid sensor molecules with fast turnover rates can be assayed for shorter times and are thus less susceptible to RNAse problems. Nucleic acid sensor

10 molecules with fast turnover can be obtained by (1) reducing the length of the incubation during the positive selection step, and/or (2) choosing fast nucleic acid sensor molecules (potentially with less favorable allosteric activation ratios) when screening individual clones emerging from the selection experiment.

Nuclease-Resistant Nucleic acid sensor molecules. By carrying out selection in

15 the presence of nucleases (*e.g.* by including depleted extracts during the negative selection step), the experiment intrinsically favors those molecules that are resistant to degradation.

Modified RNA. Covalent modifications to RNA that can render it highly nuclease-resistant are performed. Several of these modifications, including for example 2'-*O*-methylation, are compatible with hammerhead cleavage activity and are used to minimize

20 non-specific cleavage in the presence of biological samples (Usman and Blatt 2000).

Example 28. Rolling circle amplification.

A circularized nucleic acid product generated by the target-activated ligase nucleic acid sensor molecule serves as a template to promote rolling circle replication. In most

25 cases, rolling circle replication initially requires that an oligonucleotide primer be annealed to the circular nucleic acid template. This oligonucleotide primer may be provided subsequent to the nucleic acid sensor molecule reaction that generates the circular template. Alternatively, the primer may be present during the nucleic acid sensor molecule reaction as either a separate oligonucleotide or as part of the nucleic acid sensor molecule itself (Figure

30 53). Also, the primer may exist free in solution or be tethered either covalently (*e.g.*, via 5'-end modification) or non-covalently (*e.g.*, association of biotinylated primer with immobilized streptavidin or through base-pairing with complementary immobilized oligonucleotide) to a solid support. In a preferred embodiment for solid-state (*e.g.*, chip) applications, an immobilized nucleic acid sensor molecule serves directly as the primer for

rolling circle amplification as illustrated in Figure 53. Immobilization makes it possible to confine the signal generated through the rolling circle reaction to a specific spatial location, enabling the development of arrays in which each element in the array is composed of a different target-activated nucleic acid sensor molecules. In another embodiment, an

5   immobilized primer is used for rolling circle extension, also leading to a spatially localized signal. Preferably, the primer is at least 10 nucleotides long and it hybridizes to a unique site on the circular template.

Rolling circle amplification may proceed using either a DNA-directed or RNA-directed polymerase, depending upon the nature of the circular template generated by the

10   target-activated nucleic acid sensor molecules. Concatamers generated from primarily DNA templates can be generated by an enzyme preferably from the following group: Taq DNA polymerase, Tth DNA polymerase, Klenow fragment of *E. coli* DNA polymerase, bacteriophage O29 DNA Polymerase, or Bst DNA polymerase. More preferably, the polymerase enzyme is Bst DNA Polymerase. Concatamers generated from primarily RNA

15   templates can be generated by an enzyme preferably from the following group: AMV reverse transcriptase, MMLV reverse transcriptase, or Q-beta replicase. More preferably, the polymerase enzyme is MMLV reverse transcriptase.

The rolling circle amplification reaction requires a supply of nucleotide triphosphates for incorporation to generate the nucleic acid product. Quantification of the

20   rolling circle amplification reaction may be facilitated by using nucleotides containing fluorescent or radioactive labels or affinity tags as described below.

The rolling circle amplification reaction may be made to proceed exponentially by the addition of a linear oligonucleotide primer containing all or part of the circular template sequence (Figure 54). The uncircularized form of the ligase substrate can perform this

25   function. As such, in the preferred embodiment, the detection reaction is carried out with excess oligonucleotide substrate.

Detection

The rolling circle amplification reaction generates single- and double-stranded DNA. The

30   amount of the rolling circle amplification product generated can be determined by the variety of methods outlined below. The signal measured by each method can be directly related to a corresponding analyte concentration by constructing a standard curve in which a range of known concentrations of the analyte are analyzed in parallel.

Fluorescent detection. Products of the rolling circle amplification reaction may be detected fluorescently by the following methods:

a. *Intercalating dyes.* The fluorescence intensity of several dyes is known to increase upon their binding to DNA. The yield from the rolling circle amplification reaction may be measured by including one of these dyes during the reaction and using a fluorimeter to monitor the change in fluorescence intensity as the reaction proceeds. For this, a variety of nucleic acid stains is readily available (see Handbook of Fluorescent Probes and Research Products, Chapter 8, Molecular Probes, OR).

b. *Labeled nucleotides.* Fluorescent labels may be attached to the nucleotides that serve as substrates for the rolling circle amplification reaction. Fluorescent nucleotides incorporated into the newly synthesized DNA can be detected by two different mechanisms:

Homogeneous assays: nucleotides with appropriate fluorescence donor and acceptor pairs may be combined such that their sequential incorporation into a polynucleotide increases their spatial proximity and, correspondingly, the efficiency of fluorescence resonance energy transfer (FRET) (Figure 55). The resulting FRET signal may be read using a fluorimeter. The strength of this signal may be maximized by appropriate design of the ligase substrate such that its sequence contains many acceptor-donor dinucleotides. A similar process using a fluorescent UTP and a biotin-CTP derivative, followed by addition of an fluorescence-acceptor streptavidin conjugate has been described for labeling of RNA (Alpha-Bazin et al. Anal. Biochem. (2000) 286, 17-25).

Heterogeneous assays: unincorporated nucleotides may be physically removed by covalently or non-covalently capturing the rolling circle amplification reaction product (*e.g.* by pre-immobilization of the rolling circle amplification primer or by hybridization to an immobilized capture probe) and followed by washing. Fluorescent nucleotides retained with the immobilized nucleic acid product can be detected and quantified (*e.g.* using a confocal scanner) to obtain an estimate of the extent of the rolling circle amplification reaction.

Sequence-specific fluorescent hybridization probes. Hybridization of a complementary, fluorescently labeled probe with the rolling circle amplification reaction product is used in a variety of ways to generate a fluorescent signal. Strategies include (1) physical retention of a fluorescent probe by captured/immobilized RCA product, (2) co-immobilization of a

fluorophore acceptor-donor oligonucleotide pair (FRET), (3) 5'-nuclease induced cleavage of a fluorophore-quencher oligonucleotide (Taqman™) and (4) conformational changes in a fluorescent probe induced by hybridization which change its fluorescent properties. In a related aspect, the fluorescent probe may be attached to the primer that is used, leading to a
5   conformational change during transcription (e.g. Amplifluor™, Intergen, NY)

Proximity Assays: In addition to the aforementioned methods that rely on inducing physical proximity of two fluorophores through hybridization of probes with rolling circle amplification products (e.g., FRET, molecular beacons, Taqman™ etc.), other proximity-based assays may be used. This includes, but is not limited to, luminescence-based assays
10   such as Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen, Packard BioScience, CT) and Bioluminescence Resonance Energy Transfer (BRET, Packard BioScience, CT).

Radiometric read-out. By using appropriate radioactively labeled nucleotides in the rolling circle amplification reaction (e.g. alpha-P^{32}-ATP), the resulting rolling circle amplification
15   product becomes radioactive and may be quantified using instrumentation that detects radioisotope decay (e.g. phosphorimagers, x-ray film, scintillation counters). In the preferred embodiment, nucleotides provided for the rolling circle amplification reaction are labeled with tritium and the primer for the rolling circle amplification reaction is immobilized on a solid support that contains a scintillant. Co-localization of the
20   radioactively-labeled RCA product on the scintillant surface generates the signal readily detected by a scintillation counter (scintillation proximity assay, SPA).

Surface Plasmon Resonance (SPR). The primer used to initiate the rolling circle amplification reaction is immobilized on the gold surface of a detection element in an SPR detector (e.g. Biacore). As the rolling circle amplification reaction proceeds, the effective
25   mass of the primer increases, generating a signal that can be detected due to the change in surface plasmon resonance.

Coagulation: Microspheres bearing a hybridization probe complementary to the rolling circle amplification product or the primers for the rolling circle amplification reaction can lead to coagulation as the reaction progresses. Coagulation can be readily detected by means
30   that include measurement of changes in refractive index or turbidity.

Others. Rolling circle amplification is capable of generating very long polynucleotides that are very likely to lead to significant changes in the sample's physical properties. Examples include, but are not limited to, changes in viscosity, refractive index, turbidity, and

electrophysical properties such as conductivity. In general, any kind of method that can detect these chances can likely be used to monitor the rolling circle amplification reaction.

Example 29. High Throughput Screening (HTS) Assay using G protein nucleic acid
5   **sensor molecules**

Activated Gα-protein dependent nucleic acid sensor molecules are used *in vitro* to test the efficacy of the agonists and inverse agonists for any GPCR. Giα–protein cDNA can be obtained (Guthrie cDNA resource Center) and expressed in *E. coli* as His-tagged protein (Lee, Linder et al. 1994). GDP or GTP-γS are added during the purification to avoid
10   degradation. GDP-complex Giα-protein and GTP-complex Giα-protein are used to generate nucleic acid sensor molecules.

GTP-complex Giα-protein (activated-Giα-protein)-dependent nucleic acid sensor molecules are tested in a target modulation assay to screen the selected nucleic acid sensor molecules with desirable properties. For example, membrane fraction of C6 glioma cells in
15   12-well plates are loaded for 16 h in the presence and the absence of adrenaline. Cell lysate can be tested for activated Giα-protein-dependent nucleic acid sensor molecule response. Alternatively, the competitive modulation by for adenylyl cyclase is tested following the inhibition of adenylyl cyclase activity by activated Giα-protein in the presence of nucleic acid sensor molecules by a modified assay based on a previously described assay (Burt,
20   Sauté et al. 1998).

Adenylyl cyclase activity is measured as described in the presence and the absence of nucleic acid sensor molecules (Kozasa and Gilman 1995). Cell membranes from HeLa cells transfected with human cloned 5-HT$_{1A}$ receptors resuspended in buffer are used to screen compounds. The membranes are incubated with 30 M GDP and decreasing
25   concentrations of test drugs (from 100 pM to 0.1 nM) or decreasing concentrations of 5-HT, from 100 M to 0.1 nM (reference curve) for 20 min at 30°C in a final volume of about 0.5 mL (Stanton and Beer 1997). Then samples are added with GTPS and the nucleic acid sensor molecules and then incubated for a further 30 min at 30°C.

Alternatively, nucleic acid sensor molecules are generated that depend on the
30   presence of Gβ-protein uncomplexed with Gα-protein. There are four known classes of Gβ-protein. Gβ-protein complex with Gγ-protein can also play important roles in the signal transduction. After dissociation from alpha subunit, this complex is known to regulate various target protein, such as GRKs, Raf kinase, adenylyl cyclase, PLCs and ion channels.

Producing nucleic acid sensor molecules that block the complex keep the GPCR's from activating their effectors.

Example 30. Cell-based assays using GPCR nucleic acid sensor molecules

GPCR nucleic acid sensor molecules are used in cell-based assay using modifications of previously described GPCR assays (Hun, Ellington et al. 2001). For example, β-adrenergic receptor can be expressed in CHO cells. CHO cells are grown in multiwell tissue culture plates in Dulbecco's modified Eagle media (DMEM) with 10% fetal bovine serum. On the day of assay, medium is replaced with 0.2 mL treatment medium containing DMEM media containing 250 M IBMX (isobutyl-1-methylxantine) plus 1 mM ascorbic acid with test compound dissolved in DMSO. Test compounds are added at a desired concentration range (*e.g.* 10^{-9} to 10^{-4} M). Isoproterenol (10^{-5} M) is used as an internal standard for comparison of activity. Cells were incubated at 37°C on a rocker for 15-30 min. Then cells are lysed and the level of the activated Gα-protein is measured by the nucleic acid sensor molecule. The antagonist is screened in the same format in the presence of the known agonist by detecting the decreasing amount of the activated Gα-protein.

Alternatively, the agonism and the antagonism of compounds for specific or general GPCR is measured using the nucleic acid sensor molecule. For example, evaluation of compounds for antagonism can be performed using Sprague Dawley rats. The aorta from animal is isolated and freed of adhering connective tissue. Desmethylimipramine (0.1 M) and corticosterone (1 M) to block neuronal and extraneuronal uptake of noradrenaline, (±)-propranolol (1M) to block β-receptors, and yohimbine (0.1 M) to block γ-receptors are added. Aortic strips are incubated with various concentration of compounds in the presence and the absence of 10 M noradrenaline. Then the cell extract is prepared and the activated Giα-protein level in the cell is measured with the nucleic acid sensor molecules (Barlocco, Cignarella et al. 2001).

Example 31. *In vitro* assay for GPCR activation by following liberation of Gα-protein with Gα-protein dependent nucleic acid sensor molecules.

Nucleic acid sensor molecules generated against Gα-protein subunits are used to test the efficacy of agonists and inverse agonists for any GPCR *in vitro* using activated G-protein. For example, Giα–protien cDNA can be obtained (Guthrie cDNA resource Center) and expressed in E coli as His-tagged protein (Lee, Linder et al. 1994). GDP or GTP-γS are

added during the purification to avoid degradation. GDP-complex Giα-protein and GTP-complex Giα-protein are used to generate nucleic acid sensor molecules. GTP-complex Giα-protein (activated-Giα-protein)-dependent nucleic acid sensor molecule can be tested for the binding assay to screen the selected nucleic acid sensor molecules with desirable

5   properties. For example, membrane fraction of C6 glioma cells in 12-well plates were loaded for 16 h in the presence and the absence of adrenaline. Cell lysate can be tested for activated Giα-protein-dependent nucleic acid sensor molecule response. Alternatively, the competitive binding for adenylyl cyclase can be tested following the inhibition of adenylyl cyclase activity by activated Giα-protein in the presence of nucleic acid sensor molecules

10   by a modified assay based on a previously described assay (Burt, Sautel et al. 1998).

Adenylyl cyclase activity can be measured as described in the presence and the absence of nucleic acid sensor molecules (Kozasa and Gilman 1995). Cell membranes from HeLa cells transfected with human cloned 5-hydroxytryptamine 1A (5-HT$_{1A}$) receptors resuspended in buffer can be used to screen compounds. The membranes are incubated

15   with 30 mM GDP and decreasing concentrations of test drugs or decreasing concentrations of 5-HT, from 100 M to 0.1 nM (reference curve) for 20 min at 30 °C in a final volume of about 0.5 mL (Stanton and Beer 1997). Then samples are added with GTPS and the nucleic acid sensor molecules and then incubated for a further 30 min at 30°C.

Nucleic acid sensor molecules can also be generated that depend on the presence of

20   Gβ-protein uncomplexed with Gα-protein. There are four known class of Gβ-protein. Gβ-protein complex with Gγ-protein can also play important roles in the signal transduction. After dissociation from alpha subunit, this complex is known to regulate various target protein, such as GRKs, Raf kinase, adenylyl cyclase, PLCs and ion channels. Producing nucleic acid sensor molecules that block the complex keep the GPCR's from activating their

25   effectors.

Example 32 Multiplex assay for Gα proteins

Nucleic acid sensor molecules are also generated whose activity is dependent on multiple activated G-proteins. Human G-protein cDNAs are obtained from publicly

30   available databases or are cloned by RT-PCR from polka-RNA pool of appropriate source. They can be expressed as described above and use to select nucleic acid sensor molecules. The readouts for multiplex assay system are discussed above.

Example 33. Nucleic acid sensor molecules activated by native lysozyme.

Lysozyme activated nucleic acid sensor molecules lys.L1.A, B, and C are disclosed in Figure 26. Stems above the ligation junction varied in length from 2, 5 and 10 base pairs. The unimolecular NASMs were generated by PCR of the original lysozyme NASM with 5'

5 primer (TCTAATACGACTCACTATAGGACCTCGGCGAAAGC) (SEQ ID NO:296) and the following 3' primers: A ACTCTCGCTAACCTCTCTAGTCATA (SEQ ID NO:297), (B) AGTGCTCTCGCACCTAACCTCTCTAGT (SEQ ID NO:298) or (C) AGTGCGAGCCTCTCGGCTCGCACCTAACCTCTCTAGT (SEQ ID NO:299) using Taq polymerase (Invitrogen) following the manufacturer's protocol. RNAs were generated with

10 Ampliscribe T7 transcription kits following the manufacturer's protocol. RNA was heated in water for 3 min at 70 °C and then brought to room temperature before adding 5X buffer (5X buffer: 150 mM Tris, 50 mM MgCl2, pH 7.5) and lysozyme (1 uM or absent). Ligations were performed at room temperature for one hour then quenched with stop dye [formamide, 0.1 % bromophenol blue , xylene cyanol] and anaylzed on a 6% acrylamide

15 gel. Self–ligation was increased in the presence of lysozyme for constructs B and C. No ligation was observed for construct A, suggesting that the stem length above the ligation junction is critical.

This circularization can be detected using PCR across the ligation site as shown in Figure 27. Activation of (clone B is shown in Figure 28. Protocols from the supplier were

20 followed for Superscript RT and recombinant Taq Polymerase (Invitrogen). All RNA samples were reverse transcribed and PCR amplified separately with two different sets of primers. The first set was designed to amplify the total NASM RNA that was used as input into *in vitro* (TCTAATACGACTCACTATAGGACCTCGGCGAAAGC (SEQ ID NO:300) and AGTGCTCTCGCACCTAACCTCTCTAGT (SEQ ID NO:301)). The second set of

25 primers was designed to selectively amplify the RNAs that circularized (GTTGCTACAAATGATATGAC (SEQ ID NO:302)and ATGGCAATTTAGCCATGAGA (SEQ ID NO:303)). Following amplification with RT/PCR the circularized species display a ladder like pattern as a result of strand displacement. The ladder can be avoided by the use of a reverse transcriptase with RNase H activity. The gel shows in Figure 28 that after 25

30 cycles of PCR, a circularization signal appears. This signal allows for quantitation of the degree of activation of ligase in response to their target molecules.

Ligase nucleic acid sensor molecules are also able to detect their targets in cell · extracts. In Figure 29, (clone B is combined with lysozyme, and increasing amounts of HeLa cell extracts. Experiments were carried at as described for Figure 28 with HeLa cell

extract added in place of water. Even at the highest HeLa cell extract concentration, activation of clone B by lysozyme is still detectable.

Lysosyme-dependent ligase (lys11-2 from Nature Biotechnology 19, 650-655 (2001)) incubated in the presence of effector oligonucleotide 18.90a (ibid.), substrate S28A

5 (ibid.), optionally 10% cellular lysate (HeLa cells or reticulocyte lysate) and various concentrations of lysozyme at 25 °C for 1 hour, as shown in Figure 50. Ligation is observable at 100 nM and is ~30% at 1 μM. Ligation yield is unaffected by the presence of 10% reticulocyte lysate or 10% HeLa lysate.

Ligase nucleic acid sensor molecules are also able to detect their targets *in vivo*.

10 Mammalian cells were electroporated with 1, 5, 10 and 25μg RNA. The RNA was brought to a total volume of 30 ul in the presence of 20 ug salmon sperm DNA. Cells were trypsinized, counted, pelleted and resuspended in 250 ul of media. The ligase biosensor/Salmon sperm DNA mixture was added to the cells, mixed gently and incubated at room temperature for 5 minutes. The cells were electroporated and diluted in pre-

15 warmed media and incubated at ambient temperature for 5 minutes. Subsequently, the cells were plated and transferred to a 37 °C incubator and allowed to recover for 6 hours. Following recovery, the cells were trypsinized, pelleted, washed with PBS and pelleted again. Cells were resuspended in an ice cold mixture of 150 mM NaCl, 1 μl of 10% NP-40 and 6 μl 10% SDS, 28 μl0 mM Tris-HCl, pH 8.0 solution and added to a mixture of 40

20 Proteinase K (20 mg/ml). The lysate was passed through a 1ml 26G 5/8 syringe 20 times. Lysates were incubated at 37 °C for 2 hours. Following the incubation the slurry was phenol/chloroform/isoamyl alcohol extracted two times, the aqueous phase was removed and ethanol precipitated RT-PCR reactions were carried as described for figure 30. Extent of ligation and circularization was determined by RT-PCR as shown in Figure 30B, the cells

25 that were cotransfected with lysozyme showed an increased circularization.

Example 34. RT-PCR analysis of ligase nucleic acid sensor molecules/amplicon-dependent nucleic acid amplification.

RT-PCR is a quantitative method that can be used to measure the activity of a ligase

30 nucleic acid sensor molecule. As shown in Figure 46, PCR primers are used that hybridize to sequences of the ligase nucleic acid sensor molecule that are only joined after self-ligation. After ligation occurs the nucleic acid sensor molecule can be amplified, and the PCR product can be detected on an agarose gel, using ethidium bromide or other nucleic

acid staining techniques. Alternatively, the progress of the PCR reaction can be monitored using real-time detection techniques including, but not limited to, Taqman (Applied Biosystems, Foster City, CA), SYBR Green (Molecular Probes, Eugene, OR and Applied Biosystems, Foster City, CA), Scorpion (Eurogentec, Liege, Belgium), Amplifluor

5 (Serologicals, Norcross, GA) or related systems.

A specific example of a lysozyme-dependent ligase nucleic acid sensor molecule is as follows:

The ligase RNA has the sequence 5'GGACCUCGGCGAAAGCUAACGUCUCAUGGCUAAAUUGCCAUGUUGCUACAA

10 AUGAUAUGACUAGAGAGGUUAGGUGCAUCUUCAUGUCCAGUCGCUUGCAAU GCCC (SEQ ID NO:304) and is prepared through transcription off a double-stranded DNA template using T7 polymerase (Ambion MegaShortscript kit, Ambion, Austin, TX). For the ligation reaction, 200 nM nucleic acid sensor molecule RNA, 1 µM DNA RT primer 5'GGGCATTGCAAGCGACTGGACAT (SEQ ID NO:305) and 1 µM substrate oligo

15 5'ACTGAACCTGACCGTACAAAGATgcacu (SEQ ID NO:306) (DNA: upper case; RNA: lower case) are incubated with 1nM - 10 µM hen egg white lysozyme in reaction buffer (50 mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl$_2$) as previously described (Robertson MP & Ellington AD, Nature Biotechnol. 2001, 19, 650-655). After 15 min at room temperature, the reaction is stopped by diluting 300-fold with 1 mM EDTA. Of this, 5

20 µL are transferred into 25 µL of a RT-PCR reaction mix (Applied Biosystems, Foster City, CA) that contains 300 nM DNA forward primer 5'ACTGAACCTGACCGTACAAAGA (SEQ ID NO:307) and DNA reverse primer 5'TTTGTAGCAACATGGCAATTTA (SEQ ID NO:308) plus 350 nM Taqman probe 5'6FAM-CGGCGAAAGCTAACGTCTCATGG-TAMRA (SEQ ID NO:309) (Applied Biosystems, Foster City, CA). Reverse transcription is

25 performed through incubation for 30 min at 48 °C. The sample is then denatured for 10 min at 95 °C followed by 40 PCR cycles (15 sec at 95 °C, 1 min at 60 °C). The amplification process is monitored using a ABI 7000 instrument (Applied Biosystems, Foster City, CA), and threshold cycle values are calculated using the instrument software. Plotting the Ct against the logarithm of the lysozyme concentration reveals a largely linear relationship

30 (Figure 48). The linear dynamic range spans about three orders of magnitude from ~1 nM to ~1 µM. A control reaction without the addition of lysozyme gives a threshold cycle values of ~24.5. This value defines the upper limit for threshold cycle values, and is due to the small amount of product that is inevitably generated through the background ligation. On the opposite end, the lower plateau of threshold cycle values is reached with lysozyme > ~1

μM. This concentration is close to the apparent Kd (1.5 μM), and the nucleic acid sensor molecule target modulation domain is beginning to be saturated by lysozyme, triggering a reaction at maximum catalytic rate. Another specific example describes the use of the SYBR Green RT-PCR amplification detection system (Applied Biosystems, Foster City, CA). Ligation reactions are run as described above, and analysis is done through transferring 5μL of the reaction into 25 μL of a SYBR-Green RT-PCR reaction mix (Applied Biosystems, Foster City, CA) that contains 100 nM DNA forward primer 5'ACTGAACCTGACCGTACAAAGA (SEQ ID NO:310) and DNA reverse primer 5'TTTGTAGCAACATGGCAATTTA (SEQ ID NO:311). Reverse transcription is performed through incubation for 30 min at 48 °C. The sample is then denatured for 10 min at 95 °C followed by 40 PCR cycles (15 sec at 95 °C, 1 min at 60 °C). The amplification process is monitored using a ABI 7000 instrument(Applied Biosystems, Foster City, CA), and threshold cycle values are calculated using the instrument software as above (Figure 49). The threshold cycle values are directly proportional to the logarithm of the lysozyme concentration between ~1 nM to ~1 μM. A melting profile confirms that a uniform amplification product was obtained, without the appearance of artifacts such as primer-dimers.

Example 35. Ligation reactions in 3 or 2 piece systems.

Ligation reactions with varying volumes depending on the application type were performed in buffer 1, [30 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 50 μg/mL tRNA type X (Sigma)], or buffer 2 [20 mM HEPES, pH 7.4, 10% glycerol, 150 mM KCl, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 0.5 mg/ml tRNA, 2 U/μL RNaseOUT™ (Invitrogen)]. Ligation reactions performed in the presence or absence of crude cell lysates contained 200 nM or 50 nM ligase RNA, respectively. Ligase RNA was pre-annealed with a 2-fold molar excess of effector oligonucleotide by incubation for 3 min at 70 °C, followed by the addition of 5x reaction buffer at 25 °C. The appropriate concentration of protein and/or cell lysate was added to the RNA and incubated for an additional 15 min at room temperature. Ligation reactions were then initiated by the addition of this mixture to a 10x solution of the substrate oligonucleotide TK.04.82.C ACGTAGCATAGCATCGATAGCTGTTGugcacu (SEQ ID NO:357) (small letters correspond to RNA nucleotides) final concentration = 1 μM). The reactions were incubated at 25 °C, and small aliquots (2–10 μL) of reaction mix were quenched by dilution in EDTA.

Example 36. Transfection of ligase biosensors into mammalian cells by electroporation.

HEK 293, 3T3 or HeLa cells were grown in DMEM medium containing 10% fetal bovine serum to 85-90% confluency, and harvested by trypsinization. Aliquots of approximately 250,000 cells were pelleted by centrifugation (3 min at 2000 rpm), resuspended in 250 μL of growth medium, and gently mixed with 30 μL of nucleic acid mix containing 20 μg of salmon sperm DNA (carrier) and 1, 5, 10 or 25 μg nucleic acid sensor molecule ligase RNA. This mixture was incubated at room temperature for exactly 5 minutes without agitation, and transferred to electroporator cuvettes. An electric field pulse was applied to the cells at ambient temperature using a Gene Pulser II power supply (BioRad; settings: 250V, 0.975 μF high capacitance). The cells were plated in pre-warmed growth medium, incubated at 37 °C for varying periods, and harvested. Cells were washed with PBS, pelleted, and immediately subjected to total, cytoplasmic, or nuclear RNA isolation procedures or flash-frozen in liquid nitrogen and stored at -80 °C for further analysis.

Purification of total RNA from mammalian cells Pelleted cells were resuspended in ice old buffer (150 mM NaCl, 10mM Tris HCl, pH 8.0) and added to 40 μl of 10% SDS, 28 μl of 10% NP40 and 6 μl of Proteinase K (20 μg/ml). The mixture was drawn into a 1 ml syringe and passed through a 26 gauge needle at least 20 times, and then incubated at 37 °C for 2 hours. Following incubation, the slurry phenol/chloroform/isoamyl alcohol extracted two-times, the aqueous phase was removed and ethanol precipitated. The sample was 70% ethanol washed, and dried. The RNA pellets were resuspended and the yield was quantified based on OD 260. The cleanliness of the prep was assessed based on 260/280 ratio. All RNA samples were stored at -80 °C.

Example 37. Analysis of ligation and/or circularization by RT/PCR:

Protocols from the supplier were followed for reverse transcription and PCR. Either Superscript RT or AMV RT, and recombinant Taq Polymerase (Invitrogen) were used.

In order to avoid any occurrence of ligation in the detection phase of experiments, all reverse transcriptions were performed at 48 °C. All RNA samples were reverse transcribed and PCR amplified separately with two different sets of primers. First set was designed to amplify the total RNA that was used as input into *in vitro* or *ex vivo* assays. The second set of primers were designed to selectively amplify the RNAs that were ligated

to the substrate oligo (resulting in a higher molecular weight) or were ligated to their 5' end and became circularized without molecular weight change. two and three-piece ligase platforms give rise to higher molecular weight products as a result of target dependent ligation. One piece ligase platform circularize as a result of target dependent ligation.

5   When observed directly with gel based assays the circularized form has a different mobility than the linear RNA. Following amplification with RT/PCR the circularized species display a ladder like pattern as a result of strand displacement that happens in the reverse transcription phase. The ladder like pattern can be avoided by the use of a reverse transcriptase with RNase H+ activity.

10

Example 38. Preparation of extracts from mammalian cell cultures:

After stimulation with the reagent that regulates target levels, mammalian cells were rinsed briefly in cold TBS and then lysed in TBS lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% vol/vol NP-40, and 10% vol/vol glycerol; Knusel et al. 1994) or Brij Lysis

15   buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 2mM EDTA, 0.125% vol/vol NP-40, 0.875% vol/vol Brij and 10% vol/vol glycerol). Lysis buffers were supplemented with Mini Complete protease inhibitor cocktail (Boehringer Mannheim Corp.) and 1.5 mM sodium vanadate. Lysates were scraped into Eppendorf tubes and rocked for 30 min at 4 °C. Samples were then cleared by centrifugation at 10,000 x g fro 10 min at 4 °C. Protein

20   concentration was determined by the BCA assay (Pierce Chemical Co.) using BSA as a standard.

Example 39. Intron-based catalytic NASMs

Figure 61 illustrates an optical NASM derived from an intron-based catalytic

25   NASM. The optical nucleic acid sensor molecule derived from the group I intron splices *in vitro* such that it excises itself from a larger nucleic acid sequence and subsequently ligates, or joins together the two terminal portions of the RNA molecule (*i.e.*, the 5' and 3' exons). The distal termini of the 5' and 3' exons are modified to incorporate the donor and acceptor components of a FRET signaling pair. In the absence of target modulation, fluorescence

30   emission will be observed only from the donor fluorophore which is being directly excited optically. Splicing and ligation of the 5' and 3' exons brings the donor and acceptor molecules on the exons into close proximity, resulting in efficient fluorescence energy transfer (FRET) and thus a measurable increase in the fluorescence emission signal from the acceptor fluorophore (with a corresponding decrease in fluorescence emission from the

donor fluorophore). In an alternative embodiment (not shown), the acceptor molecule may be replaced with a quencher molecule (*i.e.*, an acceptor species which absorbs but does not re-radiate fluorescent energy). In this case, the splicing and ligation event will only result in a measurable decrease in the fluorescence emission signal from the donor fluorophore.

5   The thymidylate synthase ("td") intron (Construct 41)(SEQ ID NO:358) - TAATTGAGGCCTGAGTATAAGGTGACTTATACTTGTAATCTATCTAAACGGGGA ACCTCTCTAGTAGACAATCCCGTGCTAAATTGTAGGACTGCCCGGGTTCTACAT AAATGCCTAACGACTATCCCTTTGGGGAGTAGGGTCAAGTGACTCGAAACGAT AGACAACTTGCTTTAACAAGTTGGAGATATAGTCTGCTCTGCATGGTGACATGC

10   AGCTGGATATAATTCCGGGGTAAGATTAACGACCTTATCTGAACATAATG - is a group I intron sequence of 265 nucleotides in length. Other lengths and their sequences are known in the art and can used as described herein in lieu of SEQ ID NO:358 above.

An example of an intron-derived optical NASM that is modulated by target recognition is the theophylline dependent td group I intron which splices itself out of a

15   thymidylate synthase gene. The intron sequence of this optical NASM is as follows:

TAATTGAGGCCTGAGTATAAGGTGACTTATACTTGTAATCTATCTAAACG GGGAACCTCTCTAGTAGACAATCCCGTGCTAAATTGATACCAGCATCGTCTTG ATGCCCTTGGCAGCATAAATGCCTAACGACTATCCCTTTGGGGAGTAGGGTCA AGTGACTCGAAACGATAGACAACTTGCTTTAACAAGTTGGAGATATAGTCTGCT

20   CTGCATGGTGACATGCAGCTGGATATAATTCCGGGGTAAGATTAACGACCTTAT CTGAACATAATG (construct 42)(SEQ ID NO:359) wherein the bolded nucleotides represent a target modulation domain that recognizes theophylline.

The complete optical NASM comprises the intron sequence described above and the 5' and 3' exons attached thereto. Exon 1, the 5'-exon – TTTCTTGGGT (construct 43)(SEQ

25   ID NO:360) - is designed to contain a first fluorescent donor molecule, such as Cy3 (Amersham Biosciences) coupled to a nucleotide in the vicinity (*i.e.*, within no more than about 15 nucleotides) of the 3' terminus of the 5' exon. The 5'-exon length can vary with a minimum of 6 nucleotides of complementarity to P10. Exon 2, the 3'-exon - CTACCGTTTA (construct 43)(SEQ ID NO:361) - is designed to contain a second

30   fluorescent molecule, such as Cy5 (Amersham Biosciences) coupled to a nucleotide in the vicinity (*i.e.*, no more than about 15 nucleotides) of the 5' terminus of the 3' exon. The 3'- exon length can vary with a minimum of 2 nucleotides of complementarity to P10.

Intron Splicing conditions are as follows: the intron (500 nM) is heated in H₂0 to 70 °C for 3 minutes then put on ice for 1 minute. Splicing buffer (20 mM Tris-HCl, pH 7.5,

100 mM KCl, 3 mM MgCl₂), is added and the reaction is incubated on ice for an additional 15 minutes. At this time a 4.5 µl aliquot is removed for a time zero point and quenched with 5 µl stop dye (95% formamide, 20 mM EDTA, 0.5% xylene cyanol, and 0.5% bromophenol blue). GTP (50 µM) is added to the remaining reaction (4.5 µl) to start the splicing

5   reaction. The reaction is incubated at 37 °C for 1 hour and then terminated with stop dye (5 µl). The reactions are heated to 70° C for 3 minutes. Splicing of full length RNA transcripts is detected by gel analysis of labeled RNA.

The theophylline-dependent optical NASM splices *in vitro* under the same conditions as described for the intron splicing reaction, but with theophylline added to a

10   final concentration 1.5 mM in the splicing buffer. The theophylline-dependent optical NASM (500 nM) is heated in H₂0 to 70 °C for 3 minutes then put on ice for 1 minute. Splicing buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM MgCl₂) and theophylline (1.5 mM) are added and the reaction is incubated on ice for an additional 15 minutes. At this time a 4.5 µl aliquot is removed for a time zero point and quenched with 5 µl stop dye (95%

15   formamide, 20 mM EDTA, 0.5% xylene cyanol, and 0.5% bromophenol blue) (95% formamide, 20 mM EDTA, 0.5% xylene cyanol, and 0.5% bromophenol blue). GTP (50 µM) is added to the remaining reaction (4.5 µl) to start the splicing reaction. The reaction is incubated at 37 °C for 1 hour.

Target dependent modulation of the intron-derived optical NASM is followed by

20   fluorescence measurement as described herein.

Example 40. Solid-phase nucleic acid sensor array for assays with fluorescent detection—FRET chip

This example describes a general method for implementing a FRET-based

25   (fluorescence resonance energy transfer) assay utilizing nucleic acid sensor molecules. (in this case, cGMP-dependent hammerhead nucleic acid sensor molecule) wherein the nucleic acid sensor molecule is immobilized on a solid substrate, *e.g.,* within a microtiter plate well, on a membrane, on a glass or plastic microscope slide, etc.

In the specific embodiment described here, a first oligonucleotide of the nucleotide

30   sensor molecule is 3'-labeled with an acceptor or quencher fluorophore, such as TAMRA, AlexaFluor 568, or DABCYL, via specific periodate oxidation (see Example 34. for specific protocol). A second oligonucleotide of the nucleic acid sensor molecule, complementary to at least part of the first oligo portion of the NASM, is labeled with a 3' biotin and a 5' donor fluorophore, such as fluorescein (FAM, FITC, etc.). These two nucleic oligonucleotides are

heat-denatured in solution and allowed to anneal/hybridize during cooling to room temperature. After hybridization, the NASM solution is applied to a surface which has been coated with some type of avidin (streptavidin, neutravidin, avidin, etc.). This surface could include a microtiter plate well, a streptavidin-impregnated membrane, a glass or plastic

5   microscope slide, etc. In any case, the ribozyme-oligo complex is specifically immobilized via the 3' biotin on the donor oligo, leaving the binding domain free to interact with the target effector molecule.

Figure 62 shows the solution-phase FRET construct (upper panel) from which the solid-phase nucleic acid sensor molecule construct (lower panel) was derived. In the figure,

10   the species labeled 'A' and 'D' represent the acceptor and donor fluorophores, respectively; similarly, the species labeled 'F' and 'Q' represent the donor and quencher fluorophores, respectively.

As shown in Figure 62 (lower panel), the donor and acceptor fluorophores form an efficient FRET-pair; that is, upon excitation of the donor fluorophore near its spectral

15   absorption maxima, the incident electromagnetic energy is efficiently transferred (nonradiatively) via resonant electric dipole coupling from the donor fluorophore to the acceptor fluorophore. The efficiency of this resonant energy transfer is strongly dependent on the separation between the donor and acceptor fluorophores, the transfer rate being proportional to $1/R^6$, where R is the intermolecular separation. Therefore, when the donor

20   and acceptor are in close proximity, $i.e.$, a few bond-lengths or roughly 10-50 Angstroms, the fluorescent emission from donor species will be reduced relative to its output in an isolated configuration, while the emission from the acceptor species, through indirect excitation by the donor, will be detectable. Upon separation of the donor and acceptor, the donor fluorescence emission signal will increase strongly, while the acceptor emission

25   signal will show a commensurate decrease in intensity. These effects are shown in Figure 63 (upper panel) for the cGMP-dependent NASM system (SEQ ID: NO. 102).

The black arrow shown in the uppermost panel of Figure 63 indicates the site of phosphodiester bond cleavage, which occurs upon modulation fo the target modulation domain (TMD) by a cGMP, to the TMD. The melting temperature, T_m, of the remaining 5-

30   base pair sequence (GGUCU in the figure) is ~ 25 °C. Thus, after effector-mediated cleavage at room temperature, the 5-base pair cleavage fragment will rapidly dissociate from the ribozyme body and diffuse away into solution, as shown in the upper panel of Figure 63.

This target-activated nucleic acid sensor molecule system constitutes a highly sensitive real-time sensor for detecting and quantitating the concentration of the target molecule present in an unknown sample solution. The ultimate limit of detection (LOD) for this system is determined by the switch factor, defined as the ratio of the catalytic rate (in this example, the rate of cleavage) of the ribozyme sensor in the presence of its target to that of the ribozyme in the absence of its target. The dynamic range of the ribozyme sensor will be determined by the dissociation constant, K_d, for the interaction of the ribozyme binding domain with the target molecule. In theory, the effective dynamic range over which the rate-response of the NASM is linear in the target concentration has K_d as an upper bound. The lower panel of Figure 63 shows experimental data from the surface-immobilized cGMP-activated NASM. The data shown in the figure represents the FRET signal from the donor fluorophore with the sensor exposed to concentrations of 0, 100 uM, and 600 uM cGMP. The upper panel of Figure 64 shows both donor and acceptor fluorescence signals for the FRET system in the presence of 200 uM cGMP. Note that the experimental data exhibits the behavior expected, as shown in the lower panel of Figure 63. The lower panel of Figure 64 shows the donor signal form the plot in the upper panel fitted to a pseudo-first order rate equation. As shown by the closeness of the data fit, the kinetic response of this sensor system closely approximates a pseudo-first order reaction.

The measured dissociation constant for this cGMP-activated NASM with cGMP is approximately 200-500 uM. In practice, concentration measurements up to 1 mM are possible with this sensor in solution-phase measurements. The absolute precision of measurements made with this NASM will depend on the amount of background catalytic activity (*i.e.*, in the absence of target) and baseline drift of the fluorescence signals from both sample and controls due to physical factors, such as liquid handling errors, reagent adhesion, evaporation, or mixing. After some optimization, run-to-run CVs of a few percent are possible with similar FRET-based NASMs measured in solution. Immobilization of the NASM does not degrade its catalytic activity, although it may limit the effective availability of the target-binding domain for interaction with target molecules. The locally high concentration of surface-immobilized NASM will tend to offset this effect by driving the equilibrium for the association (and subsequent catalytic) reactions toward formation of ribozyme-target complex. The net result is a reduction in the observed catalytic rate of approximately 4-fold at K_d (200-500 uM) for the surface-immobilized cGMP-dependent hammerhead NASM, relative to the observed rate for the same sensor system in solution. This effect is shown in the experimental data presented in Figure 65A, which shows linear

(Panel A) and logarithmic (Panel B) plots of the observed catalytic rate constant versus cGMP concentration for the cGMP-dependent hammerhead NASM in both solution and surface-immobilized ('solid') configurations. The upper panel plots the observed rate as a function of target (cGMP) concentration on linear axes, while the lower plot plots the same

5 rate data on log-log axes. The observed rate constant (at a given concentration) for the immobilized solid-phase sensor is roughly 10-fold lower than that for the solution-phase sensor. The practical effect of this is that, while the solution-phase sensor's linear dynamic range is limited to about 0-600 uM, the solid-phase sensor's linear dynamic range is at least 2 mM. Figures 65C, 65D, and 65E shows how this general NASM strategy could be

10 extended to accomplish multiplexed detection of multiple analytes in a sample simultaneously, by using NASMs labeled with fluorophores having different emission wavelengths. Squares are NASMs immobilized on a solid. Circles are NASMs in solution. In this case, the sensors were immobilized in neutravidin-coated microtiter plate wells (96 well plate, volume of 50 uL). Detection of the fluorescent signals was accomplished in this

15 case by a microplate fluorescence reader equipped with the appropriate lamps, optics, filters, and optical detectors (PMT) manufactured by Packard Instrument Co.

This cGMP-dependent hammerhead NASM system was immobilized on streptavidin-impregnated membranes, and target-activated FRET activity observed. The generalization of this application of surface-immobilized NASM with FRET detection to a

20 micro- or macro-arrayed format on an extended substrate such as glass or plastic is easily envisioned. Such a sensor array could be used to detect and quantify the presence of an arbitrary target molecule in a complex solution, e.g., crude cell extract or biological fluid, in real time. In addition, this general NASM strategy could be extended to accomplish multiplexed detection of multiple analytes in a sample simultaneously, by using NASMs

25 labeled with fluorophores having different emission wavelengths. Experimental data for multiplexed detection using solution-phase cGMP and cAMP FRET sensors is shown in Figure 66B. This NASM, and further extensions of it to include large numbers of unique analyte-sensors, could be used for high throughput screening (HTS) in drug discovery or clonal analysis. In all of these scenarios, optical detection of the FRET signals could be

30 accomplished using a commercially available microarray imager or scanning fluorescence microscope.

The following example contains a sample protocol for the complete process of performing the solid-phase FRET assay described above, including reagent preparation,

DNA amplification, RNA transcription, RNA labeling, substrate and instrument set-up, and, finally, assay data acquisition.

Example 41. Protocol for micotiter plate-based solid-phase FRET assay:

5   The nucleic acid sensor molecules used in the assays described in Example 33. for the case of the cGMP NASM system were prepared as follows:

The appropriate sequence construct was synthesized and amplified via PCR. This was accomplished using the appropriate DNA template molecule(s) and the corresponding primers:

10   cGMP PCR template: MK.08.92.A   (SEQ ID NO:333)
5'GCTTGCAAGCCCTTAGACCCTGATGAGCCTTGCGATGCAAAAAGGTGC
TGACGACACAT CGAAACGGTGAAAGCCGTAGGTCT

Primer 1:   MK.08.66.B   (SEQ ID:334)
5' AGACCTACGGCTTTCACCGTTTCG

15   Primer 2:   MK.08.130.B   (SEQ ID:335)
5'ATACGACTCACTATAGGATGTCCAGTCGCTTGCAATGCCCTTTTAGACC
CTGATGAG

A trace amount (~ 100 ng) of template dsDNA was combined with 10-100
20   picomoles of each primer, *e.g.*, 1 uL each of 100 uM stocks. Using taq DNA polymerase, run 15 cycles of PCR using a typical (95-55-75 °C) temperature-step profile was run. The PCR products were cleaned up using a desalting/size separation column, such as a Qiagen Qiaquick PCR cleanup kit. The resuspended volume of cleaned up PCR products was ~ 35 uL. The products can be further concentrated, or added directly to a transcription reaction.

25   After synthesis and amplification of the appropriate sensor construct via PCR, the dsDNA was converted to RNA via *in vitro* transcription. This was typically accomplished using T7 RNA polymerase and pre-mixed NTPs and buffer, such as are provided in the Epicentre High Yield Transcription Kit. The transcription reaction, consisting of ~ 16-20 uL of cleaned up PCR products in a 40-50 uL total reaction volume, was typically run at 37 °C
30   for ~ 8-12 hrs. Following the transcription reaction, the products were usually purified via PAGE or using a desalting/size separation column, such as a Centrisep column (Princeton Separations).

Following production and purification of the RNA NASMs, the fluorescent acceptor labels were attached to the sensor molecules. The fluorescent donor label (in this case a

FAM label) was attached to the 5'-biotinylated capture oligo during solid-phase synthesis on a commercial DNA synthesizer, via standard phosphoramidite chemistry. The labeling procedure was accomplished by a specific oxidation of the 3' terminus of the RNA using 20 mM NaIO$_4$, followed by reaction with a hydrazide-conjugated fluorescent label. The

5   resulting covalent bond is extremely stable under the assay conditions. For the oxidation reaction, one typically 15 µL RNA (~ 200-400 pmoles in reaction), 15 µL 0.3 M NaOAc pH 5.4, and 30 µL 20 mM stock NaIO$_4$ in a were combined 1.5 mL reaction tube, which was then placed on ice in the dark for ~ 1 hr. After the oxidation reaction, the reaction products were run through a Centrisep desalting column. Alternatively the sample can be ethanol

10   precipitated and resuspended in the original volume.

The oxidized RNA uses then reacted with the dye-(in this example, AlexaFluor 568)-hydrazide conjugate. A typical reaction includes 60 µL oxidized RNA (resuspended in H$_2$O), 60 µL 0.3 M NaOAc pH 5.4, and 10 µL 20 mM stock AlexaFluor 568 hydrazide (made fresh in DMSO). This 130 uL reaction was incubated at room temperature for 2 hrs.

15   After the labeling reaction, the sample was ethanol precipitated, resuspended, gel purified (8% PAGE), and quantitated to determine concentration (using the absorbance at 260 nm). Finally, a 1 uM stock solution of the labeled NASM is prepared in water or buffer.

The substrate and plate-reader/detection system were then prepared for the assay reaction. A typical substrate was a 96 well Pierce Black Neutravidin Plate and a typical

20   plate reader was the Packard Fusion microplate reader (Packard/Perkin-Elmer). Typical settings for this instrument and this assay (as entered in the user interface menus in the instrument control software) are as follows:

- 2-channel fluorescence assay (ex:FAM*/em:FAM & TAMRA**)

25   - emission detection set to top-read

- integration time/well: 1 sec (1 read/well)

- 25 serial reads of each well, 1 min delay between reads

- lamp intensity: 10/20

- PMT voltage: 950/1100 V

30   - PMT gain: 1.0/200

*FAM excitation/emission filters: 485 nm/535 nm

**TAMRA/ or AlexaFluor 568 emission filters: 590 nm

The following reaction mixture of NASM + biotinylated capture oligo was prepared in a 1.5 mL tube:

	Reagent	Stock conc.	Final conc.
5	1 ul FAM-biotin Probe	5uM	500 nM
	1.2 ul Alexa 568 cGMP RNA	5uM	600 nM
	7.8 uL dH2O		
	10.0 uL (total volume = 10 uL x # of wells)		

The mixture was heat annealed to ~ 85 °C in 1X annealing buffer (30mM Tris, 50mM NaCl), and then cooled to room temperature. The assay reactions were run in the presence/absence of target.

To run a solid-phase FRET assay in a standard 96-well microplate, coated with neutravidin, the following procedure was used:

First, neutravidin plate surfaces were pre-blocked to prevent nonspecific adsorption of sensor molecules:

-pipet 30 ul of PBS preblocking buffer (0.05% tRNA + 0.025% Tween-20/1X PBS) into wells

-cover and shake for 15 min at room temperature on thermomixer

-rinse wells with PBS from squirt bottle

-invert plate and tap wells dry on absorbent surface

Next assay reagents were added to each well with multichannel pipet to pre-immobilized sensor molecules in plate wells:

-pipet 10 ul of annealed RNA+biotinylated probe into wells

-pipet 30 ul of PBS preblocking buffer (0.05% tRNA + 0.025% Tween-20/1X PBS) into wells & -mix by serial pipetting ~ 4x

-cover and shake for 15 min at room temperature on thermomixer

-rinse wells with PBS from squirt bottle

-invert plate and tap wells dry on absorbent surface

Next assay reaction buffers were added:

-1X HH buffer: 50 mM Tris pH 7.5, 20 mM $MgCl_2$, 0.1 mM EDTA

Finally, assay reactions were initiated and the plate was loaded into reader/detector:

-start all reactions simultaneously by adding 50 ul of cGMP in 1X HH Buffer

-shake on thermomixer for ~ 3 sec & load directly into microplate reader

5   Kinetic assay data is assigned as (target-dependent) cleavage reaction proceeds. In the case of the Packard Fusion reader described above, data points were plotted on-screen as they were acquired, giving a real-time readout as the assay proceeds.

Example 42. Real-time solid-phase nucleic acid sensor array for assays with surface
10   **plasmon resonance (SPR) detection—SPR chip:**

Surface plasmon resonance (SPR) can be used to detect target-activated ligation/cleavage events of nucleic acid sensor molecules. In SPR, a light beam is directed, via a prism or grating, onto the back surface of a thin metallic layer (typically ~ 50-100 nm
15   of gold) that has been deposited onto a glass or plastic (dielectric) substrate. The incident beam is s-polarized and directed at an angle of incidence which satisfies the condition for total internal reflection (TIR) at the metal/dielectric interface. At certain angles near the TIR critical angle, electromagnetic energy is coupled from the incident beam into surface plasmon excitation modes in the metallic layer. For a fixed excitation wavelength, the
20   precise angular position of the primary surface plasmon resonance depends very strongly on the index of refraction of the medium (typically a liquid) in contact with the front surface of the metallic layer. By monitoring the intensity of the TIR-reflected incident beam as a function of angle, the angular position of the primary surface plasmon resonance can be found. The adsorption or desorption of molecular species from the front surface of the
25   metallic layer (the SPR sensor surface) will modify the refractive index of the medium in its immediate vicinity (*i.e.*, within ~100 nm); this will result in a measurable angular shift in the position of the surface plasmon resonance. SPR is thus a sensitive technique for detecting the binding (including hybridization, etc.) and/or release of molecular species from a surface. Measurements can be performed in real time, allowing the kinetic
30   characterization of binding events, as shown in Figure 67 panels C + D. A system utilizing a large collimated incident beam and an area detector (such as a CCD detector) could be used to generate SPR images of two-dimensional arrays of surface-immobilized species, such as nucleic acid sensor molecules.

The demonstration of an SPR assay format utilizing nucleic acid sensor molecules was accomplished by coupling hammerhead (HH) nucleic acid sensor molecules (Figure 67, Panel B) to the native gold surface of a commercially available integrated single-channel (i.e., non-imaging) SPR module, called SPReeta (see Figure 67, Panel A). The SPReeta

5   modules are manufactured by Texas Instruments, Inc., and marketed by Nomadics, Inc. (Stillwater, OK). The SPReeta is an integrated package comprised of an optical plastic housing, within which is embedded a light emitting diode (LED) with a polarizer, two reflecting surfaces, i.e., the SPR surface and a mirror, and a linear diode array detector, where the mirror directs the TIR-reflected beam onto diode array. The entire package is

10   built onto a printed circuit board (PCB), with standard dual inline pins which allow the sensor module to be plugged directly into another PCB containing preamplifier and signal processing circuits. The real-time processed data is sent to a computer (PC) via a standard serial port connector. A software application logs, analyzes, and displays the data to the user.

15   Numerous immobilization schemes for nucleic acid sensor molecules are possible on the gold SPR surface substrate: (1) direct attachment to the gold SPR surface of a construct with a closed stem I, and extended 3' and 5' termini on an open stem III, via a terminal 5' gamma-thiol GTP incorporated during transcription (see Figure 68); (2) direct attachment to a passively adsorbed neutravidin-on-gold layer on the SPR surface using constructs with a

20   truncated, open stem I, and a closed stem III, post-transcriptionally modified by specific periodate oxidation of the 3' terminus, followed by subsequent reaction with biotin-XX-hydrazide to produce a 3' biotinylated hammerhead nucleic acid sensor molecule (see Figure 69); (3) indirect attachment to the gold SPR surface via hybridization of a nucleic acid sensor molecule to a thiol-modified capture oligo via a 3' terminal extension on an

25   open stem I, with stem III closed (see Figure 70). The latter two strategies are flexible in terms of application, allowing easy thermal or chemical stripping and recharging of the sensor surface with nucleic acid sensor molecules in situ, whereas direct attachment of the nucleic acid sensor molecules via incorporated thiol-groups would limit the sensor surface to a single use, or require the use of extremely harsh stripping agents.

30   All of the above nucleic acid sensor molecule constructs were synthesized and/or amplified from existing PCR products. Synthetic DNA templates were prepared by standard solid-phase methods and were purified by denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). RNAs were generated by in vitro transcription of the appropriate DNA templates that were generated by PCR.

Standard *in vitro* transcription reactions were conducted in a total volume of 50 μl containing ~20 pmol template DNA, 50 mM Tris-HCl (pH 7.5 at 23 °C), 10 mM MgCl$_2$, 50 mM dithiothreitol (DTT), 20 mM spermidine, 2 mM each of the four ribonucleoside 5′-triphosphates (NTPs), and 35 units/μl T7 RNA polymerase (T7 RNAP) by incubation at

5   37°C for 2 h. 5′-thiol-modified RNA was prepared by *in vitro* transcription wherein GTP was replaced by guanosine 5′-O-(3-thiotriphosphate) (Sigma Chemical Co., St. Louis, MO). 3'-biotinylatedRNA was prepared by post-transcriptionally modification by specific periodate oxidation of the 3' terminus, followed by subsequent reaction with biotin-XX-hydrazide. The resulting RNA products were purified by denaturing 10% PAGE and

10   isolated from the gel by elution with 10 mM Tris-HCl (pH 7.5 at 23 °C), 200 mM NaCl, and 1 mM ethylenediamine tetraacetic acid (EDTA). The recovered RNA was precipitated with ethanol, resuspended in deionized water (dH$_2$O), and stored at –20 °C until use. In addition, the thiol-modified DNA capture oligo was synthesized and purified.

       Initial surface immobilization experiments using a kinased (5' 32P-labeled) sample

15   of the thiol-modified DNA capture oligo were conducted on Au/Cr coated microscope slides in order to optimize the thiol-gold coupling procedure. Initial results indicated that blocking of the surface with mercaptohexanol (MCH) and control of pH during the surface coupling reaction were critical. Good surface coupling and binding capacities were observed.

20   The passive adsorption of neutravidin to the gold sensor surface (via cysteine residues) was investigated, prior to subsequent attachment of a biotinylated hammerhead nucleic acid sensor molecule. Purified neutravidin (Pierce, Rockford, IL) was acquired and flowed over the SPReeta sensor surface at a concentration of 50-100 ug/mL. The adsorption of the neutravidin was monitored in real time. cGMP and cAMP hammerhead nucleic acid

25   sensor molecules were generated, amplified, and transcribed with a truncated, open stem I, and a closed stem III. The constructs were then post-transcriptionally modified by specific periodate oxidation of the 3' terminus, followed by subsequent reaction with biotin-XX-hydrazide. These constructs allowed for a maximal cleavage fragment (~ 65 nt=21.5 kD), and thus maximal dynamic range in the observed SPR signals, with only 5 nt retained on

30   the surface. As with the capture oligo and direct-attachment thiol constructs, the melting temperature, Tm, of the base-paired region retaining the ribozyme body after cleavage is only ~ 25 °C, facilitating rapid dissociation and removal from the SPR sensor surface upon effector-induced cleavage.

After characterizing the SPR signature and kinetic parameters for neutravidin adsorption, the surface immobilization of 3'-biotinylated cGMP nucleic acid sensor molecules (see Figure 71, Panel A). Initial experiments showed a significant reduction in RIUs upon introduction of excess (1 mM) effector solution was introduced into the flow cell

5   (see Figure 71, Panel B). For a 65 nt mass change, the maximum expected signal change (*i.e.*, for complete cleavage of all surface-immobilized nucleic acid sensor molecules) was ~ 2145 RIU; the observed signal change was ~ 1487 RIU, with a signal to noise ration (SNR) > 100. Subsequent negative control experiments (using cAMP) confirmed the effector-dependent origin of the observed signal. The cleavage The cleavage/dissociation time

10   course was fitted to a pseudo-first order rate function (correlation coefficient = 0.9977), with an observed rate constant of ~ 1 per minute.

The usual scheme for attachment of the nucleic acid sensor molecule to the capture oligo and subsequent target - induced activity is shown in Panel C of Figure 71. In order to verify the generality of the surface attachment strategy, as well as the catalytic activity of

15   surface-immobilized nucleic acid sensor molecules, the 3'-biotinylated cGMP-dependent hammerhead was immobilized via neutravidin to a SPREETA sensor chip surface. The cGMP HH Riboreporter surface was then exposed to a range of concentrations of cGMP (0-1 mM) and varying Mg^{2+} concentration in the ambient buffer (2-20 mM). Target-modulated cleavage was observed for Mg^{2+} concentrations > 10 mM, and cGMP concentrations

20   greater than ~ 10 uM. The time-course for a portion of this type of experiment is shown in Figure 72. This data was generated using a single surface-adsorption step with neutravidin, followed by sequential addition of increasing concentrations of cGMP, with buffer flushes and/or active buffer pumping (peristaltic) in between each cGMP addition. All sample fluids were injected at volumes of ~ 25-40 uL, while buffer washes were ~ 100 uL of 1X buffer.

25   For active pumping, volume exchange was ~ 500 uL of 1X buffer.

Example 43. Solid-phase nucleic acid sensor array for assays with fluorescent detection—ligase chip

Nucleic acid sensors can be tethered to solid supports while still maintaining their

30   activity, as shown in Figure 66. The plot in Panel B of Figure 66 shows the fraction of this endonuclease-based nucleic acid sensor molecule that is released by self-cleavage over time in presence of its analyte. When exposed to its target analyte, the solid-phase nucleic acid sensor molecule increased in activity. In the case of ligase based NASMs, the sensor molecule ligated itself to a labeled oligonucleotide in the presence of target, and the

detected signal increase. In the case of the endonucleolytic nucleic acid sensor, the molecule cleave a labeled oligonucleotide from itself, resulting in a decrease in the deleted signal.

These ligase based nucleic acid sensor molecules can be used in solid-state
5   scintillation proximity assays, membrane assays, chip-based assays with fluorescent detection chip based assays with electrochemical detection, and in assays using intrinsic (non labeled detection such as, surface plasmon resonance. Figure 74 presents the two principal solid-phase array (chip) formats used for ligase-based nucleic acid sensors. (a) In the capture format, the ligation reaction in the presence of target is performed *in vitro*,
10 followed by an optional amplification step, hybridization of the reaction products (amplified or not) to a capture oligo array, and finally washing and detection/readout of the array. Here, each capture tag oligonucleotide is a unique sequence address, allowing spatial multiplexing of the assay over numerous different NASM/target combinations. (b) In the *in situ* format, the ligase sensors are pre-immobilized on the chip via unique capture tags; the
15 ligation reaction in the presence of target is performed *in situ* on the chip surface, followed by washing and detection/readout of the array. Under ordinary solution-phase reaction conditions for ligase-based nucleic acid sensors, a external effector oligo (the non-immobilized analog of the capture oligo used to immobilize the sensor to a surface) is typically present. In order for the capture chip assay format (*i.e.*, *in vitro* reaction followed
20 by on-chip capture and readout, as shown in Figure 74A) to be a viable the case where no isothermal amplification step is performed prior to hybridization, it is necessary either to (a) exclude such a effector oligo form the *in vitro* reaction, so that the appropriate portion of the sensor sequence would be free to hybridize to the surface-immobilized capture oligo in the subsequent capture step; or (b) to include some form of effector oligonucleotide that could
25 be easily separated or competitively displaced from the sensor molecule after the *in vitro* reaction, allowing hybridization to the surface-immobilized capture oligo in the subsequent capture step. Both strategies were shown to be viable. In particular, Figure 73 shows an example, of an ERK-dependent ligase-based nucleic acid sensor system ligase-based NASMs can function effectively in solution-based assays without the presence of a effector
30 oligonucleotide. The ligation signals from 2 separate reactions that were run in the presence of target (ERK) and the presence and absence of an external effector oligonucleotide, respectively; showed *i.e.*, that the reaction products amplified equally in the same number of PCR cycles, that the sensor functions equally well in the presence or absence of a capture effector oligonucleotides. A parallel result is shown for the case of the reactions run in the

absence of target (ERK) and the presence and absence of an external passivating capture oligo, respectively. The results, which have been shown to be valid for all ligase-based sensor systems studied (> 10 different ligase sensor systems), is that the ligase-based sensors function in solution-based reactions equally well in the presence or absence of a

5 effector oligo. The *in vitro* reaction steps for all capture chip data shown and referred to in the following examples were all conducted in the absence of a effector oligo.

For chip-based assays, the activity of the nucleic acid sensor molecules can be detected using many methods shown schematically in Figure 14. In one embodiment of the invention, the nucleic acid sensor molecule has a self cleaving activity. These nucleic acid

10 sensors can be attached to a solid substrate in such a way that cleavage results in the release into solution of a nucleic acid fragment of known size. This fragment can then be detected through the use of agarose gels using stains that bind to nucleic acids, including ethidium bromide. The activity of the nucleic acid sensor molecule with self-cleaving activity could also be detected through attaching a fluorophore and a quencher group to either side of the

15 cleavage site, as detailed above, and in Figure 62. In another embodiment of the invention, the nucleic acid sensor molecule has ligation activity. The components of a ligase-based nucleic acid sensor molecule immobilized to a chip surface are shown in Figure 73. The activity of this sensor in the presence of target molecules can be detected through fluorescence, chemiluminescence, radioactivity, or electrochemical means. The labeling of

20 the sensor can be accomplished either during the assay reaction, during a subsequent amplification reaction (*e.g.*, via RT), or after the sensor (as a component of the assay reaction products) has been captured to a microarray chip for read out, as shown in Figure 74.

Figure 75 shows a multiplex *in situ* ligase sensor chip, with pre-immobilized

25 radiolabeled sensors activatable by lysozyme (LYS) and FMN. The left panel shows the pre-hybridized sensor-substrate complexes, in this case immobilized via a biotin linker on the substrate oligo on a streptavidin/glass chip. The right panel shows the array after it has been exposed to target mixtures consisting of lysozyme only, FMN only, and lysozyme plus FMN, as indicated in the figure. Following the *in situ* ligation reaction, the chip was

30 subjected to a denaturing wash, in order to remove any sensor molecules not ligated to their substrates. Note the retention of labeled sensors only in spots that had been exposed to the corresponding target, and that both protein (lysozyme) and small molecule (FMN) sensors function in the presence of nontarget analytes.

Figure 13 shows a solid-phase nucleic acid sensor molecule array on a streptavidin membrane. In this case, 6 different ligase sensors were immobilized on a streptavidin membrane, and assayed in an *in situ* format, with each NAMS being reacted with an analyte mixture containing all 6 targets. The plot shows the detected retention of ligation signal

5   following target exposure and washing. Here detection was accomplished using a radiolabeled sensor, with the ligase immobilized via a biotin tag on the 5' terminus of the substrate oligo

Figure 76 shows dose-response data for ERK-dependent ligase-based nucleic acid sensor molecules. The data plotted in the left panel was generated from a PAGE-based

10   assay, while the data in the right panel was generated from a capture chip assay (right panel). The reaction incubation time for both assays was approximately 2 hrs. Detection in both cases was via radiolabeled sensors and substrate oligonucleotides. Note that the capture chip assay recapitulates the apparent affinity constant (Kd) of ~ 1 uM measured from the gel-assay data, indicating that the chip mode of capture and detection accurately reflects all

15   reaction parameters observed in the *in vitro* case.

Figure 77 shows dose-response data for an *in situ* ligase-based nucleic acid sensor molecule array populated with ERK-dependent unlabeled ligase sensors. The left panel shows an image of the *in situ* chip after exposure to target (ERK) plus radiolabeled substrate and subsequent washing to remove unligated substrate. The reaction incubation time for

20   both assays was approximately 2 hrs. Each array spot corresponds to a different concentration of target, giving rise to the observed pattern of positive/negative controls (10/0 uM, respectively), and the concentration profile (0-10 uM) shown. The retained ligation signal from each spot in the concentration profile is plotted vs. its corresponding target concentration in the right panel. The linear dose-response observed here is a result of

25   two effects: (1) the surface-immobilization of the NASM reduces its effective catalytic rate over the rate observed *in vitro*; and (2) the much higher surface to volume (S/V) ratio in the *in situ* chip format, relative to the *in vitro* reaction/capture chip format, has been shown to promote nonspecific interactions of protein targets (*e.g.*, ERK) with the chip surface coating. This leads to a reduced effective target concentration in the reaction volume at each

30   sensor spot.

Figure 78 shows the dose-response data for a ERK-dependent ligase-based nucleic acid sensor molecule capture array. The NASMs in this case were fluorescently 3' labeled with Cy3-labeled ligase sensors, and the substrate oligos were 5' labeled with biotin. After 2 hr. *in vitro* reactions in the presence of varying concentrations of target (ERK), the reaction

products were hybridized to the capture array. After hybridization and washing, tyramide signal amplification (TSA) was performed using a streptavidin-conjugated horseradish peroxidase (HRP) pulse Cy5-labeled tryramide HRP substrates. The captured and amplified fluorescent ligation signal for each spot is plotted in the right panel vs. its corresponding

5   target concentration. This data demonstrates the high sensitivity of fluorescence detection for ligase-based nucleic acid sensor molecule arrays in both *in situ* and capture formats.

Figure 79 shows the components of a generalized construct for an amplifiable ligase-based nucleic acid sensor molecule. Each sensor in an array would have a unique 3' capture tag sequence domain, and a common RT primer binding site. The external substrate

10   oligonucleotide contains a signal generation label, which may comprise a direct label (*e.g.*, fluorescent, radionuclide, enzyme-linked, etc.) or an affinity tag (*e.g.*, biotin) for subsequent indirect labeling or amplification (*e.g.*, via TSA), or a specific sequence (*e.g.*, the T7 RNA polymerase promoter sequence) which will facilitate selective amplification of ligated sensors via RT or RT-PCR. The amplification process (RT or RT-PCR) can also be used to

15   incorporate multiple direct or indirect labels or affinity tags, thus increasing the sensitivity of the sensor system. In one embodiment, detection can be accomplished by using a chimeric (RNA/DNA) external substrate oligonucleotide which contains a DNA sequence that is necessary for subsequent amplification of the nucleic acid, such as the T7 RNA polymerase promoter sequence, as shown in Figure 79. An arbitrary number (*e.g.*, 10, as

20   shown) of unique analyte-specific sensors are each outfitted (*e.g.*, via PCR) with an arbitrary number (*e.g.*, 10, as shown) of unique sequence capture tags. In the present example, the result would be a panel of 100 uniquely addressed sensors. Thus, ten different targets could be simultaneously assayed under ten different conditions, with the reaction products RT-amplified (and simultaneously labeled with multiple fluorophores per sensor

25   molecule) and spatially sorted by hybridization to a capture array chip, and finally detected using standard techniques, such as fluorescence. Upon ligation of such an external substrate, RT can be performed to synthesize a complementary strand to the sensor, as shown in Figure 81. A given NASM is reacted *in vitro* with its corresponding target analyte and a chimeric (*i.e.*, containing both DNA and RNA bases) substrate oligo containing the

30   T7 promoter sequence comprised of DNA bases. Following the ligation reaction, an RT step is performed to synthesize a complementary cDNA strand to the RNA sensor molecule. The NASM is then hydrolyzed or degraded with RNaseH. In the case of NASM that ligated its substrate in the presence of target, this leaves the cDNA strand with the DNA T7 promoter sequence annealed. This complex forms a suitable platform for the T7 RNA polymerase to

transcribe multiple copies of those cDNA sequences corresponding to sensors which were triggered by their respective targets. During transcription, NTPs with direct fluorescent labels or affinity tags (*e.g.*, biotin) are multiply incorporated into the transcribed cRNA molecules. After hybridization to the capture array, the amplified cRNA sensors can be

5   detected via their direct labels, or by subsequent staining with a conjugate to the incorporated affinity tags, *e.g.*, streptavidin-phycoerythrin. After hydrolysis of the original RNA sensor strand, the double-stranded DNA portion containing the T7 promoter sequence will allow *in vitro* transcription of the cDNA to cRNA by standard methods. This transcription step will amplify the sensor sequence, and can be used to simultaneously

10   incorporate nucleotides that contain direct or indirect (*e.g.*, biotin, or other affinity tag) labels for subsequent fluorescence- or luminescence-based detection, as shown in Figure 80. This detection strategy can be implemented in a multiplexed format by using PCR to incorporate arbitrary sequence capture oligo domains, as well as common RT primer binding domains, to a panel of ligase-based sensors. In particular, Figure 80 shows a

15   generalized strategy for performing a multiplexed capture chip formatted assay with ligase-based nucleic acid sensors. An arbitrary number (*e.g.*, 10, as shown) of unique analyte-specific sensors are each outfitted (*e.g.*, via PCR) with an arbitrary number (*e.g.*, 10, as shown) of unique sequence capture tags. In the present example, the result would be a panel of 100 uniquely addressed sensors. Thus, 10 different targets could be simultaneously

20   assayed under 10 different conditions, with the reaction products RT-amplified (and simultaneously labeled with multiple fluorophores per sensor molecule) and spatially sorted by hybridization to a capture array chip, and finally detected using standard techniques, such as fluorescence. In one embodiment, shown in Figure 80 , a generalized strategy for performing a highly sensitive capture chip formatted assay with ligase-based nucleic acid

25   sensors. A given sensor molecule is reacted *in vitro* with its corresponding target analyte and a chimeric (RNA) substrate oligo containing the T7 promoter sequence comprised of DNA bases. Following the ligation reaction, an RT step is performed to synthesize a complementary cDNA strand to the RNA sensor molecule. The RNA sensor molecule is then hydrolyzed or degraded with RNAseH. In the case of a sensor which ligated its

30   substrate in the presence of target, this leaves the cDNA strand with the DNA T7 promoter sequence annealed. This complex forms a suitable platform for the T7 RNA polymerase to transcribe multiple copies of those cDNA sequences corresponding to sensors which were triggered by their respective targets. During transcription, NTPs with direct fluorescent labels or affinity tags (*e.g.*, biotin) are multiply incorporated into the transcribed cRNA

molecules. After hybridization to the capture array, the amplified cRNA sensors can be detected via their direct labels, or by subsequent staining with a conjugate to the incorporated affinity tags, *e.g.*, streptavidin-phycoerythrin.

Highly multiplexed *in vitro* or *in situ* assays can then be performed, using the specific hybridization of each sensor's unique capture oligo to address its signal to a feature spot whose position on the array is known. The presence or relative concentration of a given analyte can then be determined by the presence/absence of a signal in the expected array position, and the relative concentration determined by comparison of the relative signal intensity to that of other sensor spots in the array.

Listed below are selected DNA template sequences that can be used to generate ligase-based nucleic acid sensor molecules via PCR, as described above:

Synthetic DNA universal templates for 2- & 3-piece ligase constructs (underlined regions = primer annealing regions)

ERK.A.LIG.TMPLT (DG.20.58A):   SEQ ID NO: 109
5'<u>CTATAGGACTTC</u>GGCGAAAGCCGTTCGACCACGCTAAGGAGGATTTCC
GAAAGCGGCTACGGTCCGCCAGTGTCTTAGACAG<u>GAGGTTAGGTGC</u>

ERK.E.LIG.TMPLT (DG.20.58E):   SEQ ID NO: 111
5'-
<u>CTATAGGACTTC</u>GGCGAAAGCCGTTCGACCAGCTAAGGAGGATTTCCGAAAGC
GGCTACGGTCCGCCAGCTCTTAGACAG<u>GAGGTTAGGTGC</u>

LYS.LIG.TMPLT.L1-11.2 :   SEQ ID NO: 336
5'<u>CTATAGGACTTC</u>GGCGAAAGCTAACGTCTCATGGCTAAATTGCCATGT
TGCTACAAATGATATGACTAGA<u>GAGGTTAGGTGC</u>

FMN.LIG.TMPLT.L1-R7C13:   SEQ ID: 337
5'<u>CTATAGGACTTC</u>GGTCCAGTGCTCGTGCACTAGGCCGTTCGACCTTCAG
GATATGCTTCGGCAGAAGGGAACTTAGACAG<u>GAGGTTAGGTGC</u>3'

THEO.LIG.TMPLT.L1-D1:   SEQ ID: 338
5'<u>CTATAGGACTTC</u>GGTCCAGTGCTCGTGCACTAGGCCGTTCGACCATGAT
ACCAGCATCGTCTTGATGCCCTTGGCAGCATCTTAGACAG<u>GAGGTTAGGT
GC</u>

For 3-piece system:

5'-primer: TK.16.32.A   SEQ ID: 339

5'-TTCTAATACGACTCA<u>CTATAGGACTTC</u>

3'-primer (5 nt sub): TK.16.32.B   SEQ ID: 340

5'-ATTCGAGATGTCCTTGGACCAAAGCC<u>GCACCTAACCTC</u>

3'-primer (15 nt sub):TK.16.32.15NT   SEQ ID NO: 341

5'-ATTCGAGATGTCCTTGGACCAAAGCCTCCATCGTGC<u>GCACCTAACCTC</u>

Substrate (5 nt):   TK.04.82.A   SEQ ID NO: 342

5'-Biotin/Fluor-CATGCGACCTTACGATCAGATGACCTugcacu

Substrate (15 nt):   MK.08.125A   SEQ ID NO: 343

5'-Biotin-TCCATCGTGCGCACu

<u>For 2-piece system:</u>

5' primer: TK16.32A   SEQ ID NO: 344

5'-TTCTAATACGACTCA<u>CTATAGGACTTC</u>

3' primer: MK.08.125B   SEQ ID NO: 345

5'-TCCATCGTGC<u>GCACCTAACCTC</u>

Substrate: MK.08.125A   SEQ ID NO: 346

5'-Biotin-TCCATCGTGCGCACu

Example 43. Conversion of nucleic acid sensor molecules into unimolecular ligase NASMs

The Erk and pERK dependent nucleic acid sensor molecules can be converted to unimolecular ligase NASMs. The engineered NASM ERK B, ERK CW45-33-C08 and ERK CW45-33-D09 were converted by PCR as described for Figure 28 with primers ·GGACTTCGGCGAAAGC and AGTGCTCTCGCACCTAACCTCCTGTCT (SEQ ID NO:363). NASMs pERK CW45-33-C04, CW45-33-D05 and CW45-33-H03 were all converted with primers GGACTTCGGCGAAAGC (SEQ ID NO:364) and AGTGCTCTCGCACCTAACCTCCTGTCT (SEQ ID NO:365). Circularization can be detected by RTPCR for ERK B (GCTACGGTCCGCCAGTTCTT) (SEQ ID NO:366) and CGCTTTCGGAAATCCTCCTT) (SEQ ID NO:367), ERK CW45-33-C08 (GCTACGGTCCGCCAGGGGCT (SEQ ID NO:368) and CGCTTTCGGAAATCCTCCTT (SEQ ID NO:369)), ERK CW45-33-D09 (GCTACGGTCCGCCAAAAGCT (SEQ ID NO:370) and CGCTTTCGGAAATCCTCCTT (SEQ ID NO:371)) and pERK CW45-33-C04, CW45-33-D05 and CW45-33-H03 (AAGGGGAAAGCGTTATTAAG (SEQ ID NO:372) and TCGAGGAACCAATTCGCTAG (SEQ ID NO:373)).

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Variations, modifications, and other implementations of what is described herein

30   will occur to those of ordinary skill in the art without departing from the spirit and scope as

claimed. Accordingly, the invention is to be defined not by the preceding illustrative

description but instead by the spirit and scope of the following claims.

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We claim:

1. A nucleic acid sensor molecule comprising

    (a) a target modulation domain, wherein said target modulation domain recognizes a target molecule;

    (b) a linker domain; and

    (c) a catalytic domain,

wherein said nucleic acid sensor molecule comprises an optical signal generating unit.

2. The nucleic acid sensor molecule of claim 1, wherein said optical signal generating unit includes at least one signaling moiety.

3. The nucleic acid sensor molecule of claim 1, wherein said optical signal generating unit comprises at least a first signaling moiety and a second signaling moiety.

4. The nucleic acid sensor molecule of claim 3, wherein said first and second signaling moieties change proximity to each other upon recognition of a target by the target modulation domain.

5. The nucleic acid sensor molecule of claim 4, wherein said first and second signaling moieties comprise a fluorescent donor and a fluorescent quencher, and recognition of a target by the target modulation domain results in an increase in detectable fluorescence of said fluorescent donor.

6. The nucleic acid sensor molecule of claim 4, wherein said first signaling moiety and said second signaling moiety comprise fluorescent energy transfer (FRET) donor and acceptor groups, and recognition of a target by the target modulation domain results in a change in distance between said donor and acceptor groups, thereby changing optical properties of said molecule.

7. The nucleic acid sensor molecule of claim 1, wherein said optical signal generating unit consists essentially of a first signaling moiety, wherein said first

signaling moiety changes conformation upon recognition of a target by the target modulation domain, thereby resulting in a detectable optical signal.

8. The nucleic acid sensor molecule of claim 1, wherein said nucleic acid sensor molecule includes at least one modified nucleic acid.

9. The nucleic acid sensor molecule of claim 1, wherein said catalytic domain comprises an endonucleolytic ribozyme.

10. The nucleic acid sensor molecule of claim 9, wherein said endonucleolytic ribozyme is a *cis*-endonucleolytic ribozyme or a *trans*-endonucleolytic ribozyme.

11. The nucleic acid sensor molecule of claim 9, wherein said endonucleolytic ribozyme is a hammerhead ribozyme.

12. The nucleic acid sensor molecule of claim 1, wherein said catalytic domain comprises a self-ligating ribozyme.

13. The nucleic acid sensor molecule of claim 12, wherein said self-ligating ribozyme is a *cis*-ligase ribozyme or a *trans*-ligase ribozyme.

14. The nucleic acid sensor molecule of claim 12, wherein said self-ligating ribozyme is a 1-piece ligase, 2-piece ligase or 3-piece ligase.

15. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a target selected from the group consisting of proteins, post-translationally modified forms of proteins, peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, ions, carbohydrates, polysaccharides, hormones, receptors, antigens, antibodies, viruses, metabolites, co-factors, drugs, dyes, nutrients, and growth factors.

16. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a target selected from the group consisting of a protein and a post-translationally modified protein.

5   17. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a target that is a post-translationally modified protein, and wherein the post-translation modifications are selected from the group consisting of: phosphorylation, prenylation, glycosylation, methionine removal, N-acetylation, acylation, acylation of cysteines, myristoylation, alkylation,

10   ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycoslylation, deamination of glutamine and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation, and lipidation.

18. The nucleic acid sensor molecule of claim 1, wherein said target is a
15   protein kinase.

19. The nucleic acid sensor molecule of claim 1, wherein said target is a phosphorylated protein kinase.

20   20. The nucleic acid sensor molecule of claim 19, wherein said phosphorylated protein kinase is a monophosphorylated protein kinase or a diphosphorylated protein kinase.

21. The nucleic acid sensor molecule of claim 18, wherein said protein is
25   ERK.

22. The nucleic acid sensor molecule of claim 19, wherein said post-translationally modified protein is pp ERK.

30   23. The nucleic acid sensor molecule of claim 18 wherein said protein kinase is ERK1 or ERK2.

24. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a component of a MAP kinase pathway, a product of

a MAP kinase pathway, a MAP kinase pathway associated protein, or an extracellular component of a MAP kinase pathway.

25. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a component of the ERK1/2 MAP kinase pathway.

26. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a component of the JNK MAP kinase pathway.

27. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a component of the P38 MAP kinase pathway.

28. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes an endogenous form of a MAP Kinase (MEK), an endogenous form of a MAP Kinase Kinase (MEKK), or an endogenous form of MAP Kinase Kinase Kinase, (MEKKK).

29. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes an endogenous form of RAF kinase.

30. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a Ras protein.

31. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a phosphatase.

32. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a GTP binding protein.

33. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a GPCR.

34. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a cytokine.

35. The nucleic acid sensor molecule of claim 1, wherein said target modulation domain recognizes a growth factor.

5

36. The nucleic acid sensor molecule of claim 1, wherein said target recognition domain recognizes a cellular metabolite.

37. The nucleic acid sensor molecule of claim 1, wherein said target recognition domain recognizes a small molecule.

10

38. The nucleic acid sensor molecule of claim 1, wherein said nucleic acid sensor molecule comprises RNA, DNA, or both RNA and DNA.

39. A composition comprising the nucleic acid sensor molecule of any one

15   of claims 1-38 or claims 74-99 and a buffer.

40. A composition comprising the nucleic acid sensor molecule of any one of claims 1-38 or claims 74-99 and a tissue extract, a cell extract or an *in vitro* cell culture.

20

41. The composition of claim 39 or 40, further comprising an RNase inhibitor.

42. The composition of claim 41, wherein said RNase inhibitor is selected

25   from the group consisting of Va-riboside, vanadyl, tRNA, polyU, RNaseIn and RNaseOut.

43. The composition of claim 39 or 40, wherein said composition is substantially RNase-free.

30

44. A composition comprising at least one nucleic acid sensor molecule according to any one of claims 1-38 or claims 74-99, affixed to a substrate.

45. The composition of claim 44, wherein said substrate is glass, gold or other metal, silicon or other semiconductor material, nitrocellulose, nylon, or plastic.

46. The composition of claim 44, wherein the nucleic acid sensor molecule is covalently attached to said substrate.

47. The composition of claim 44, wherein the nucleic acid sensor molecule is non-covalently attached to said substrate.

48. The composition of claim 44, wherein the nucleic acid sensor molecule is immobilized to the substrate via hybridization of a terminal portion of the nucleic acid sensor molecule to an oligonucleotide that is bound to the surface of the substrate.

49. The composition of claim 44, wherein said composition comprises a plurality of nucleic acid sensor molecules immobilized to the substrate via hybridization of a terminal portion of the nucleic acid sensor molecule to an array of oligonucleotides bound to the substrate at spatially discrete regions.

50. The composition of claim 44, wherein at least two members of said plurality each recognize different target molecules.

51. The substrate of claim 44, wherein said substrate comprises at least 50 nucleic acid sensor molecules.

52. The substrate of claim 44, wherein said substrate comprises at least 250 nucleic acid sensor molecules.

53. The substrate of claim 44, wherein said substrate comprises at least 500 nucleic acid sensor molecules.

54. The substrate of claim 44, wherein said substrate comprises at least 5000 nucleic acid sensor molecules.

55. A system for detecting a target molecule comprising a composition according to any one of claims 41-54, and a detector in optical communication with said composition, wherein said detector detects changes in the optical properties of said composition..

5

56. The system of claim 55, further comprising a light source in optical communication with said composition.

57. The system of claim 56, further comprising a processor for processing

10   optical signals detected by the detector.

58. The system of claim 55, wherein said system comprises a plurality of nucleic acid sensor molecules, wherein at least two of said biosensor molecules each recognize different target molecules.

15

59. A method of identifying or detecting a target molecule in a sample, the method comprising:

contacting a sample suspected of containing a target molecule with a nucleic acid sensor molecule according to any one of claims 1-38 or claims 74-99, wherein a

20   change in the signal generated by the optical signal generating unit indicates the presence of said target in said sample.

60. The method of claim 59 further comprising quantifying the change signal generated by the optical signal generating unit to quantify the amount of

25   target molecule in the sample.

61. The method of claim 59 or 60 wherein the sample is selected from the group consisting of: environmental samples, biohazard materials, organic samples, durgs and toxins, flavors and fragrances, and biological samples.

30

62. The method of claim 59 or 60 wherein the sample is a biological sample, including cells, cell extracts or lysates, tissues or tissue extracts, bodily fluids, serum, blood and blood products.

63. The method of claim 59 or 60 wherein the target is selected from the group consisting of proteins, post-translationally modified forms of proteins, peptides, nucleic acids, oligosaccharides, nucleotides, metabolites, drugs, toxins, biohazards, ions, carbohydrates, polysaccharides, hormones, receptors, antigens,

5   antibodies, viruses, metabolites, co-factors, drugs, dyes, nutrients, and growth factors.

64. The method of claim 59 or 60 wherein the target is selected from the group consisting of proteins and post-translationally modified forms of proteins.

10

65. The method of claim 64, wherein said target is a post-translationally modified protein, and wherein the post-translation modifications are selected from the group consisting of: phosphorylation, prenylation, glycosylation, methionine removal, N-acetylation, acylation, acylation of cysteines, myristoylation, alkylation,

15   ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycoslylation, deamination of glutamine and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation, and lipidation.

66. The method of claim 59 or 60 wherein the target is a protein kinase.

20

67. The method of claim 59 or 60, wherein said target is a phosphorylated protein kinase.

68. A diagnostic system for identifying or detecting a target molecule, the

25   diagnostic system comprising

a nucleic acid sensor molecule according to any one of claims 1-38 or 74-99; and

a detector in communication with said nucleic acid sensor molecule, wherein said detector detects changes in the signal generated by the optical signal generating

30   unit of said nucleic acid sensor.

69. The diagnostic system of claim 68, further comprising a processor for processing signals detected by the detector.

70. A method of identifying or detecting a protein kinase in a sample, the method comprising:

contacting a sample suspected of containing a protein kinase with a nucleic acid sensor molecule according to claim 1, wherein said nucleic acid sensor

5   molecule has a target recognition domain that recognizes a protein kinase, wherein a change in the signal generated by the optical signal generating unit indicates the presence of protein kinase in said sample.

71. The method of claim 70, further comprising quantifying the amount of

10   signal generated by the optical signal generating unit to quantify the amount of protein kinase in the sample.

72. A method of identifying a modulator of protein kinase activity, the method comprising:

15   contacting a test agent with a protein kinase and nucleic acid sensor molecule according to claim 1, wherein said nucleic acid sensor molecule has a target recognition domain that recognizes a protein kinase, wherein recognition of the protein kinase by the target recognition domain of said nucleic acid sensor molecule results in a change in the signal generated by the optical signal generating unit and

20   further wherein changes in the signal generated by the optical signal generating unit in the presence and absence of said test agent indicates the test agent is a modulator of said protein kinase activity.

73. The method of claim 70 or 72, wherein the catalytic domain of said

25   nucleic acid sensor molecule comprises a *cis*-ligase ribozyme or a *trans*-ligase ribozyme.

74. A nucleic acid sensor molecule comprising:

a target modulation domain that recognizes ERK;

30   a catalytic domain that comprises a ligase or cis-hammerhead; and

a linker domain that links said target modulation domain and said catalytic domain.

75. A nucleic acid sensor molecule comprising:

a target modulation domain that recognizes phosphoERK;

a catalytic domain that comprises a ligase or a cis-hammerhead; and

a linker domain that links said target modulation domain and said catalytic

domain.

5

76. A nucleic acid sensor molecule comprising:

a target modulation domain that recognizes lysozyme;

a catalytic domain that comprises a 1-piece cis-ligase; and

a linker domain that links said target modulation domain and said catalytic

10   domain.

77. A nucleic acid sensor molecule comprising:

a target modulation domain that recognizes any one of cCMP, cAMP, or

cGMP;

15   a catalytic domain; and

a linker domain that links said target modulation domain and said catalytic

domain, wherein said nucleic acid sensor molecule comprises an optical signal

generating unit or a non-radioactive detectable label.

20   78. The nucleic acid sensor molecule of any one of claims 74-76 wherein the

nucleic acid sensor molecule comprises an optical signal generating unit.

79. The nucleic acid sensor molecule of any one of claims 74-76, wherein

said nucleic acid sensor molecule comprises a detectable label.

25

80. The nucleic acid sensor molecule of claim 79, wherein the label is a

radioactive label.

81. The nucleic acid sensor molecule of claim 79, wherein the radioactive

30   label is ^{32}P, ^{33}P, ^{14}C, ^{35}S, ^{3}H, or ^{125}I.

82. The nucleic acid sensor molecule of any one of claims 74-77, wherein

said nucleic acid sensor molecule comprises a fluorescent label.

83. The nucleic acid sensor molecule of claim 82, wherein the fluorescent label is fluorescein, DABCYL, or a green fluorescent protein (GFP) moiety.

84. The nucleic acid sensor molecule of claim 77 or claim 78, wherein the optical signal generating unit comprises a fluorescent moiety and a quenching moiety, wherein recognition by the target modulation domain causes causes a change in detectable fluorescence by the optical signal generating unit.

85. The nucleic acid sensor molecule of any one of claims 74-77, wherein said nucleic acid sensor molecule comprises an enzymatic label.

86. The nucleic acid sensor molecule of any one of claims 74-77, wherein said nucleic acid sensor molecule comprises an affinity capture tag label.

87. The nucleic acid sensor molecule of claim 74, wherein the target modulation domain recognizes ERK1, ERK2 or both.

88. The nucleic acid sensor molecule of claim 74, wherein the target modulation domain and the catalytic domain are as shown in any one of SEQ ID NO. 80 and the linker is randomized.

89. The nucleic acid sensor molecule of claim 74, wherein the target modulation domain and the catalytic domain are as shown in any one of SEQ ID NOS. 47, 118 and 119 and the linker is randomized.

90. A nucleic acid sensor molecule that recognizes ERK comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 90-95, 108-116, 131-133, 140-295, 349, 351, and 356.

91. A nucleic acid sensor molecule that recognizes phospoERK comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 5-8, 37-39, 44-45, 81-89, 96-100, 121-130, 352, and 353.

92. A nucleic acid sensor molecule that recognizes any one of cCMP, cAMP or cGMP comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 40-43, 103, and 135-139.

93. A nucleic acid sensor molecule that recognizes lysozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 46, 47, 76, 105-107.

94. A 1-piece ligase ribozyme comprising

a target modulation domain that recognizes a target;

a linker domain;

a catalytic domain,

wherein the 5' and 3' ends of the ligase ligate to each other upon recognition of the target by the modulation domain.

95. A 2-piece ligase ribozyme comprising

a target modulation domain that recognizes a target;

a linker domain;

a catalytic domain comprising an oligonucleotide substrate ligation site and and oligonucleotide supersubstrate binding domain, wherein upon recognition of the target by the modulation domain, the 3' end of the an oligonucleotide supersubstrate is ligated to the 5' end of the oligonucleotide substrate ligation site.

96. A 3-piece ligase ribozyme comprising

a target modulation domain that recognizes a target;

a linker domain;

a catalytic domain comprising comprising an oligonucleotide substrate binding domain capable of binding an oligonucleotide substrate and an effector-oligonucleotide binding site capable of binding an effector oligonucleotide, wherein upon recognition of the target by the modulation domain, and in the presence of binding of the effector oligonucleotide to the effector-oligonucleotide binding site, then the 3' end of the oligonucleotide substrate is ligated to the 5' end of the ligase.

97. A 1-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 47, 105-107, 119.

98. A 2-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 347, 349, and 351.

99. A 3-piece ligase ribozyme comprising the nucleic acid sensor molecule shown in any one of SEQ ID NOS. 46, 75, 76, 108-116, 118, 121-130, and 352.
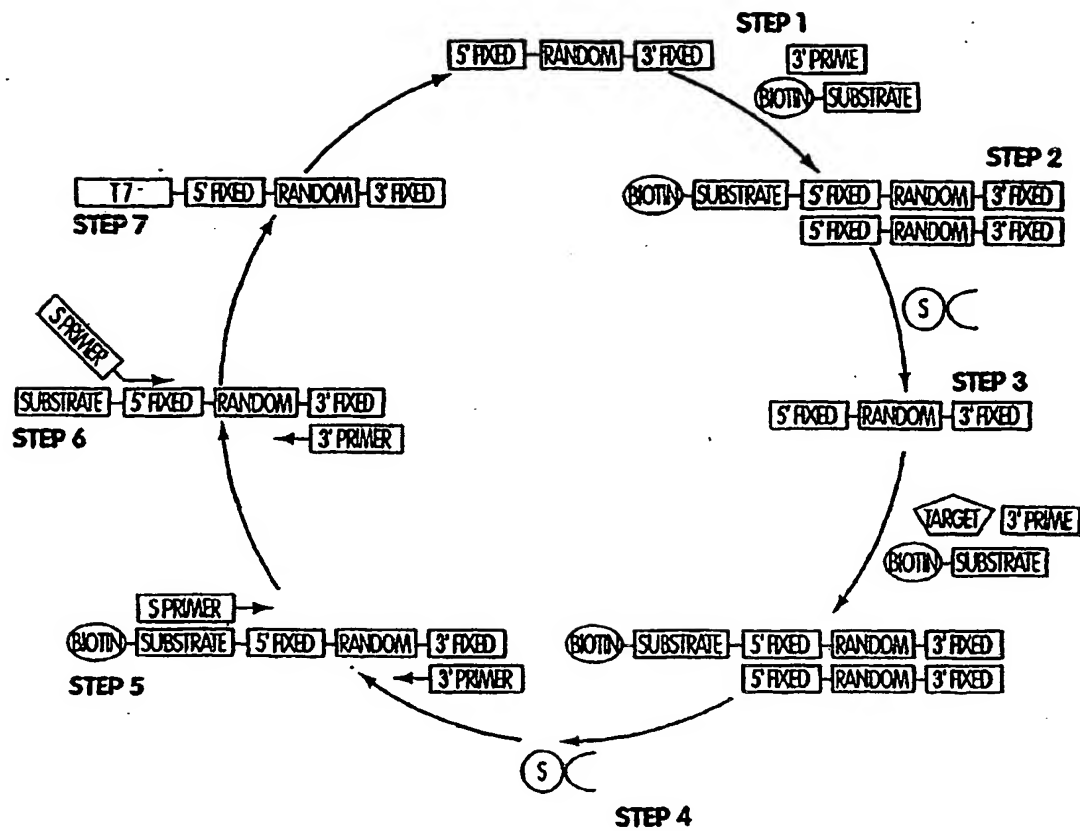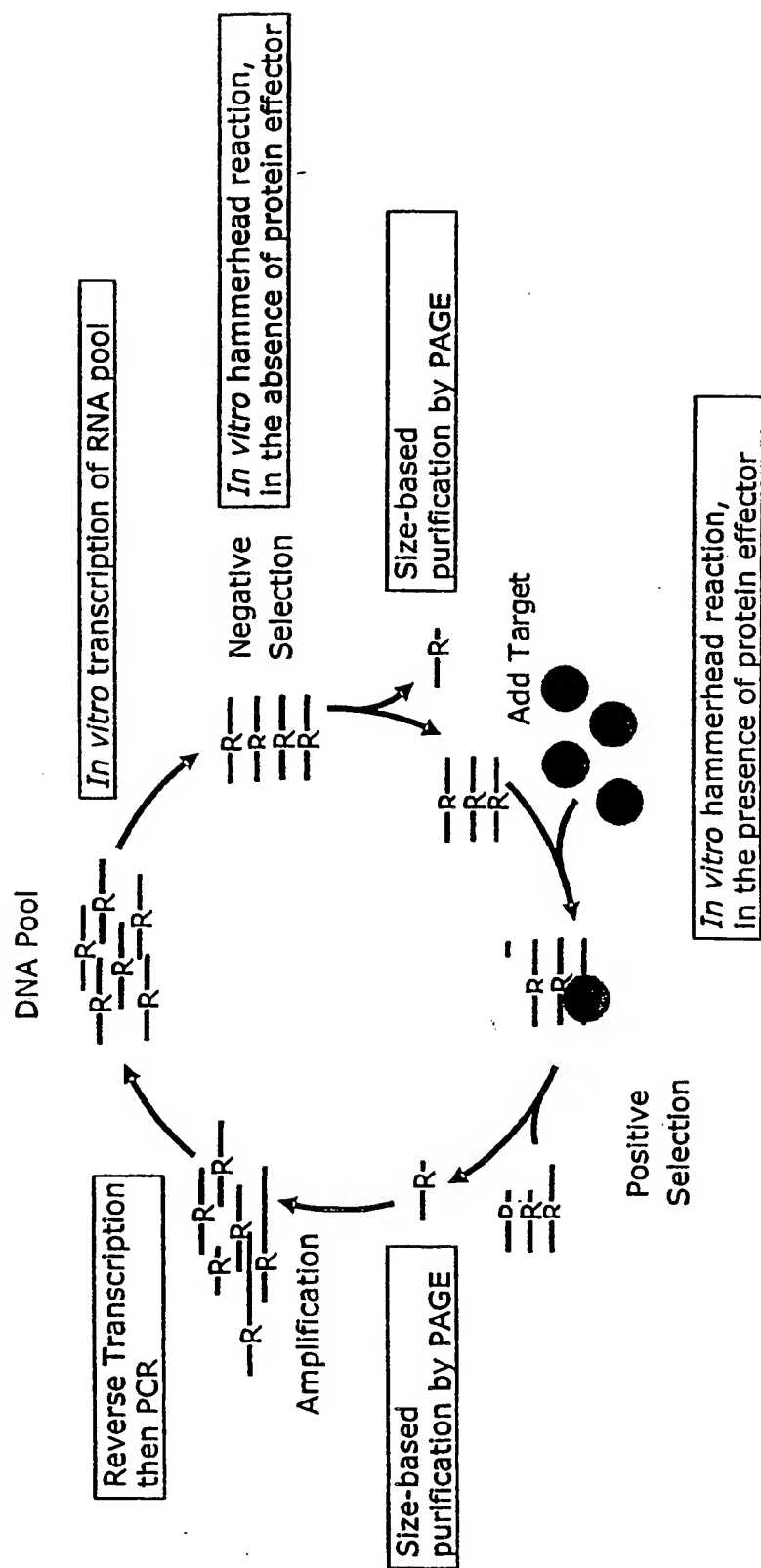
5

10

FIGURE 1A

Figure 1B

SEQ ID NO:51

A

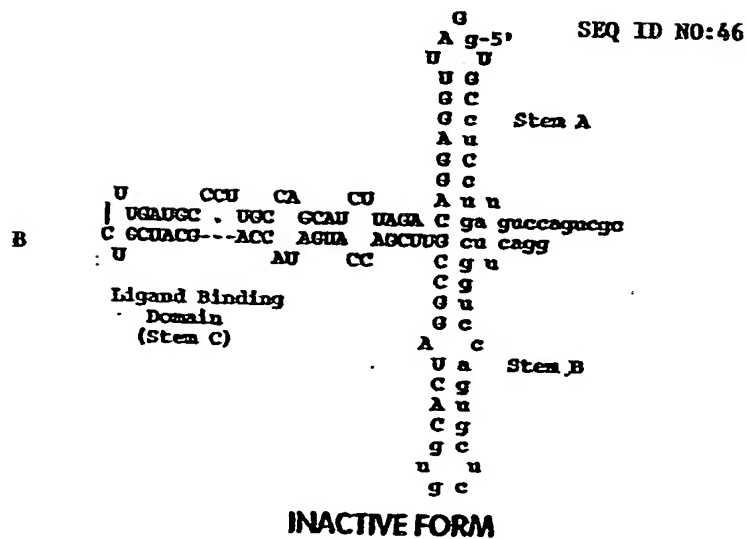GCGACTGGACATCACGAG   tag
3'-cgcugaccuguagugcuc U /   SEQ. ID NO: 46
                       C G   Substrate
                       G C
                       U A
                       G C
                       G U
                       A g-5'
                       U g
                       U a
                       G c
                       G u
                       A u   Stem A
                       G C
                       G
                       A C
U   CCU   CA   CU C
| UGAUGC . UGC GCAU UAGA C
C GCUACG---ACC AGUA AGCUUG |
: U   AU   CC C g
                       C g
                       G u
                       G c
Ligand Binding   A   c
    Domain   U a   Stem B
   (Stem C)   C g
                       A u
                       C g
                       g c
                       u   u
                       g c

ACTIVE FORM

                       G
                       A g-5'   SEQ ID NO:46
                       U U
                       U G
                       G C
                       G c   Stem A
                       A u
                       G C
                       G c
U   CCU   CA   CU A u n
| UGAUGC . UGC GCAU UAGA C ga guccagucgc
C GCUACG---ACC AGUA AGCUUG cu cagg
: U   AU   CC C g u
                       C g
                       G u
                       G c
Ligand Binding   A   c
   Domain   U a   Stem B
  (Stem C)   C g
                       A u
                       C g
                       g c
                       u   u
                       g c

INACTIVE FORM

FIGURE 2

3'- D-ccuguagugcuc
                    C
                    C
                    U
                    G
                    G
                   [A] g-F5'
                   [U] g
                    U a
                    G c
                    G u
                    A C
         U   CCU CA   CU G
       | UGAUGC . UGC GCAU UAGA C   SEQ ID NO:47
       C GCUACG---ACC AGUA AGCUUG C
         U   AU   CC C g
                            C g
                            G u
                            G c
                            A   c
                            U   a
                            C   g
                            A   u
                            C   g
                            g   c
                            u   u
                            g   c

UNQUENCHED LIGAND-BOUND FORM

A

                      G
                    [A] g-5'
                    [U] U
                     U G
                     G c
                     G c
                     A u
                     G c
                     G c
         U   CCU CA   CU A u
       | UGAUGC UGC GCAU UAGA C ga gucc(D)
       C GCUACG---ACC AGUA AGCUUG cu cagg(F)
         U   AU   CC C g u   SEQ ID NO:47
                            C g
                            G u
                            G c
                            A   c
                            U   a
                            C   g
                            A   u
                            C   g
                            g   c
                            u   u
                            g   c

B

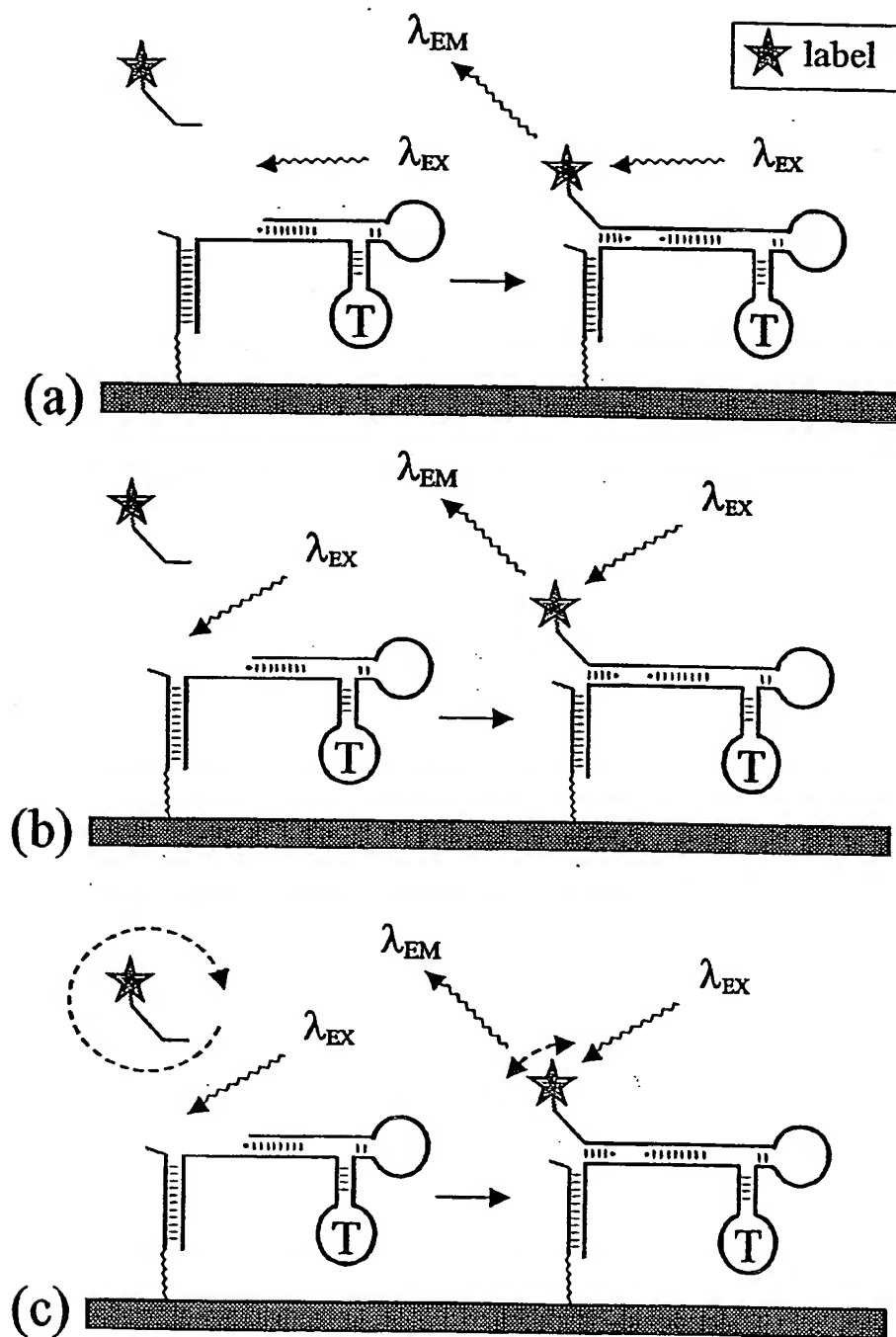**QUENCHED NO-LIGAND FORM**

FIGURE 3

FIGURE 4
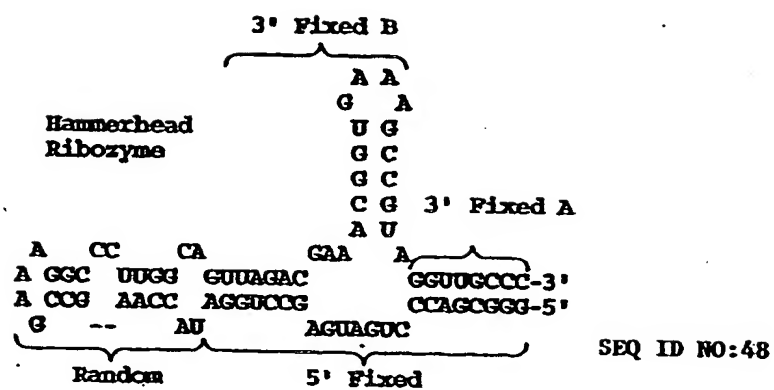
FIGURE 5

A
```
      A    CC      CA
    A GGC  UUGG  GUGGUAU -D          SEQ ID NO:49
    A CCG  AACC  A-F.
    G   --     AU
```

UNQUENCHED LIGAND-BOUND FORM

B
```
      CC      GA
    G  UUGGC  UGGUAU-D              SEQ ID NO:49
    G  AGCCG  ACCAUA-F
    AA       -A
```

QUENCHED NO-LIGAND FORM

FIGURE 6

CELLULAR
MEMBRANE

RECEPTOR

DRUG
TARGET

SIGNAL
TRANSDUCTION
CHAIN

NUCLEAR MEMBRANE

TRANSCRIPTION FACTOR

DNA

PHYSIOLOGICAL
FUNCTION
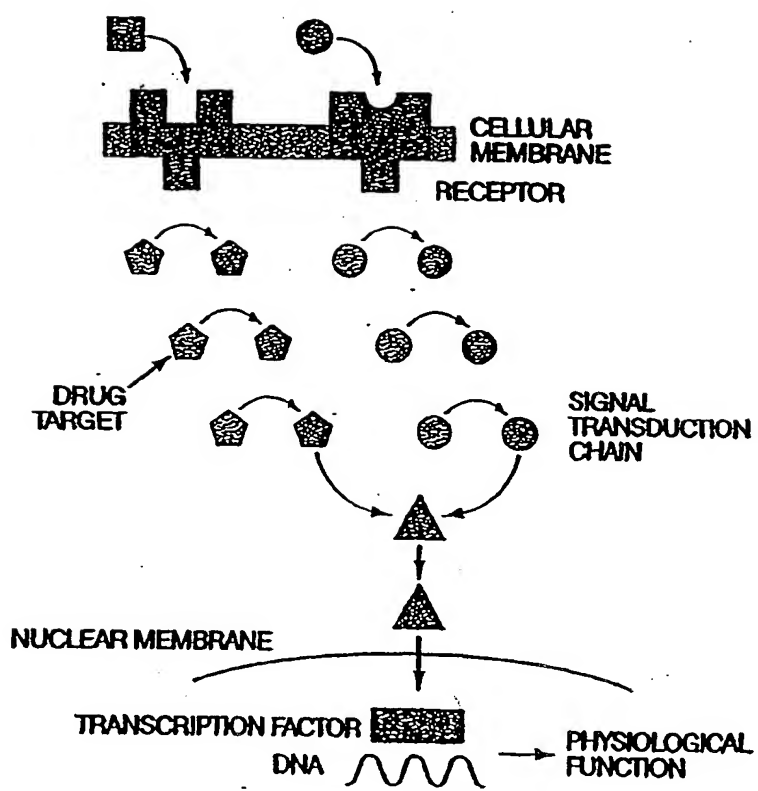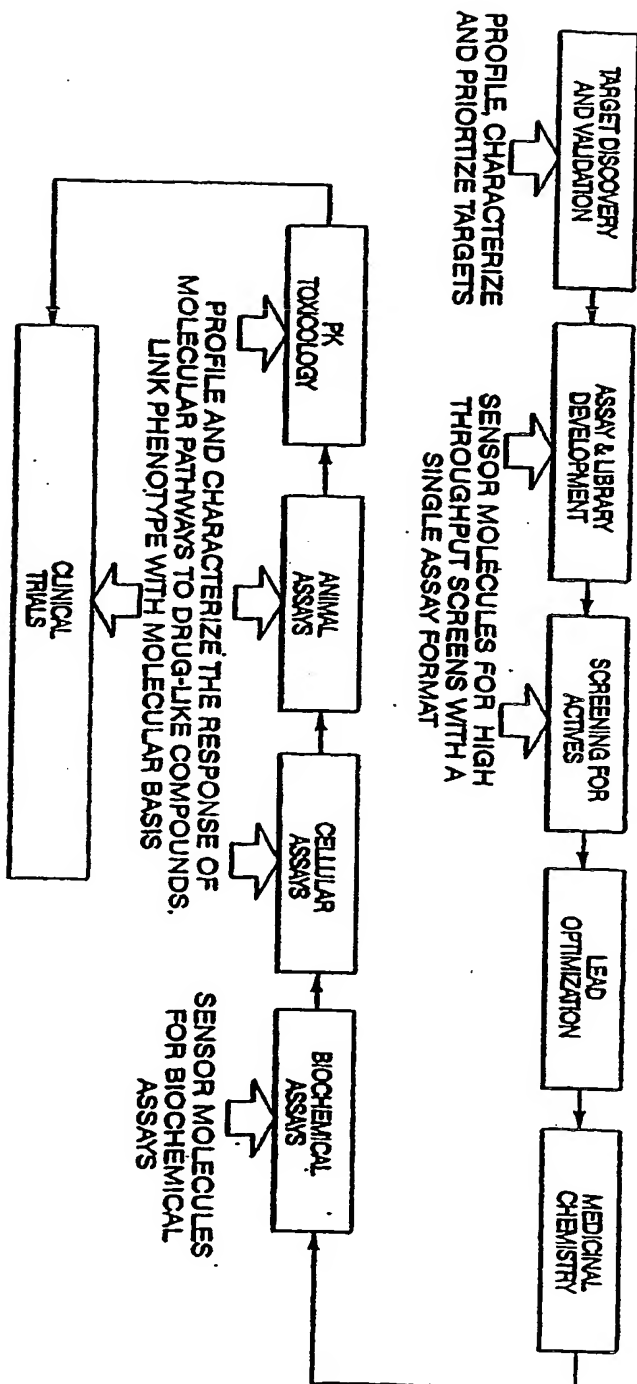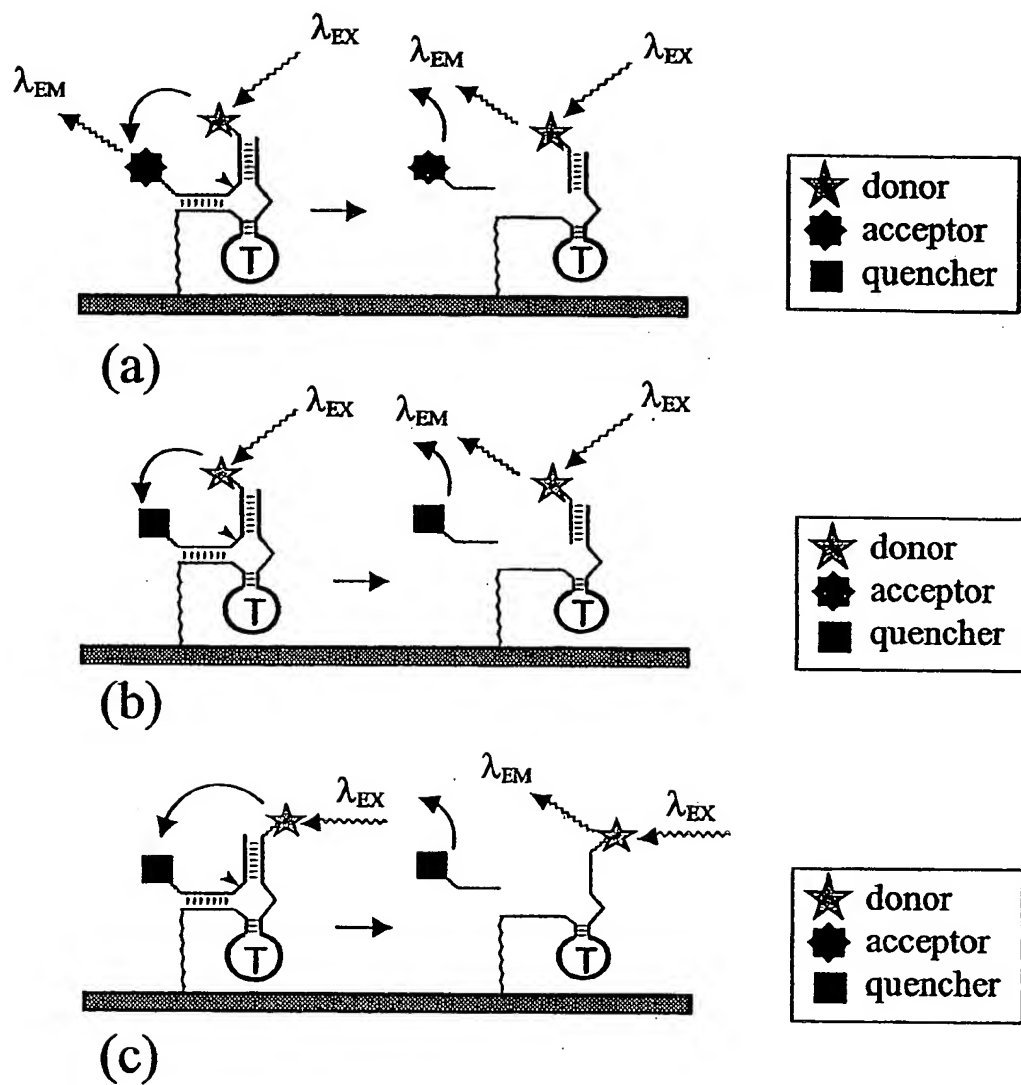
FIGURE 7

FIGURE 8

Figure 9.

Figure 10.
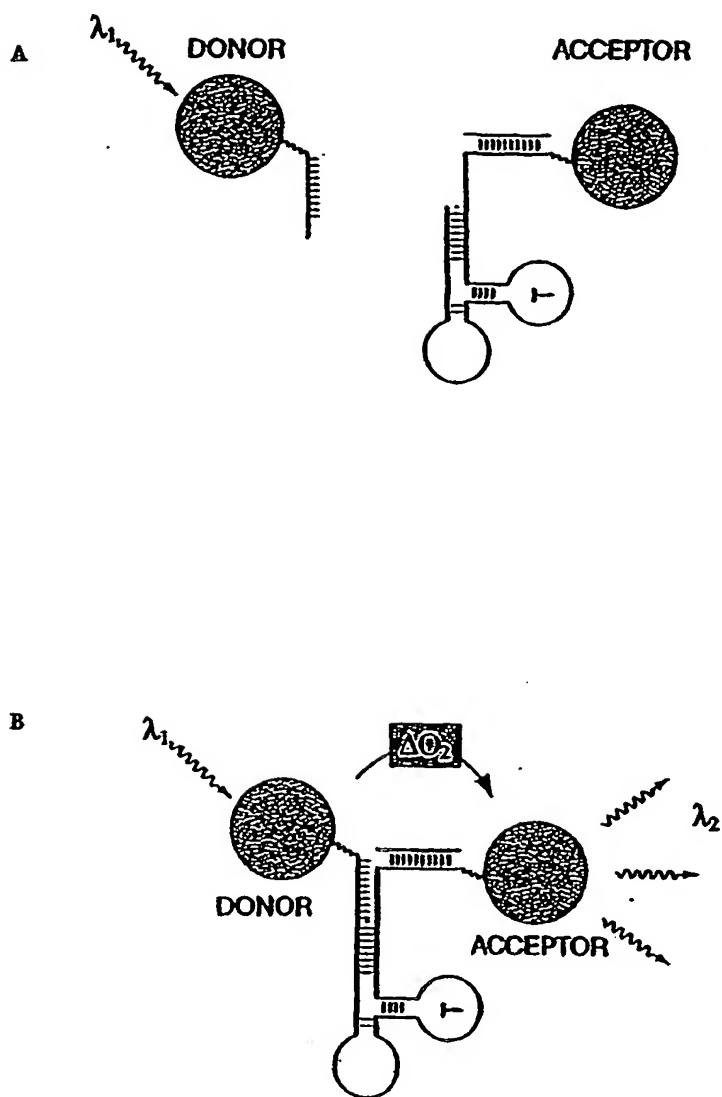
FIGURE 11
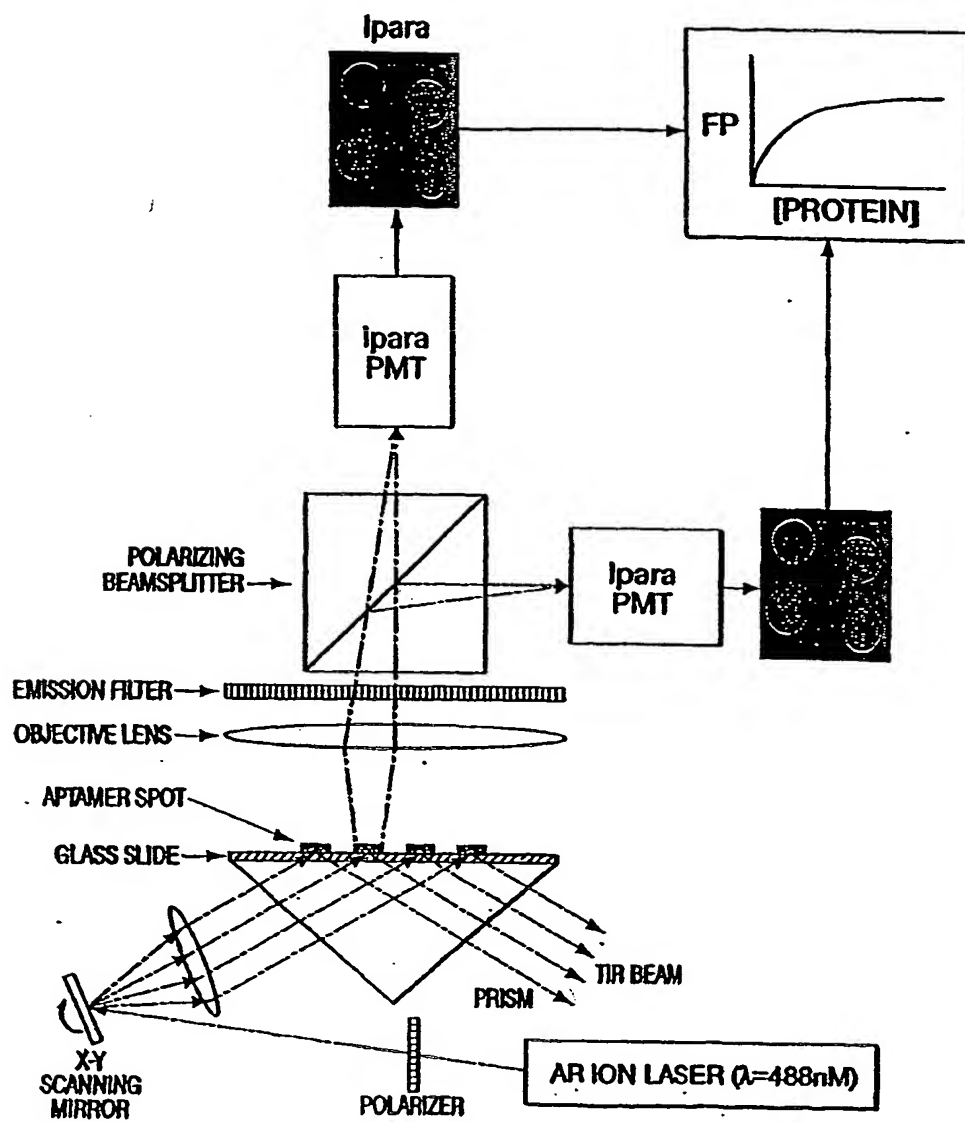
FIGURE 12

Figure 13
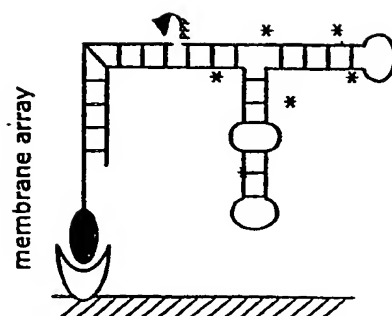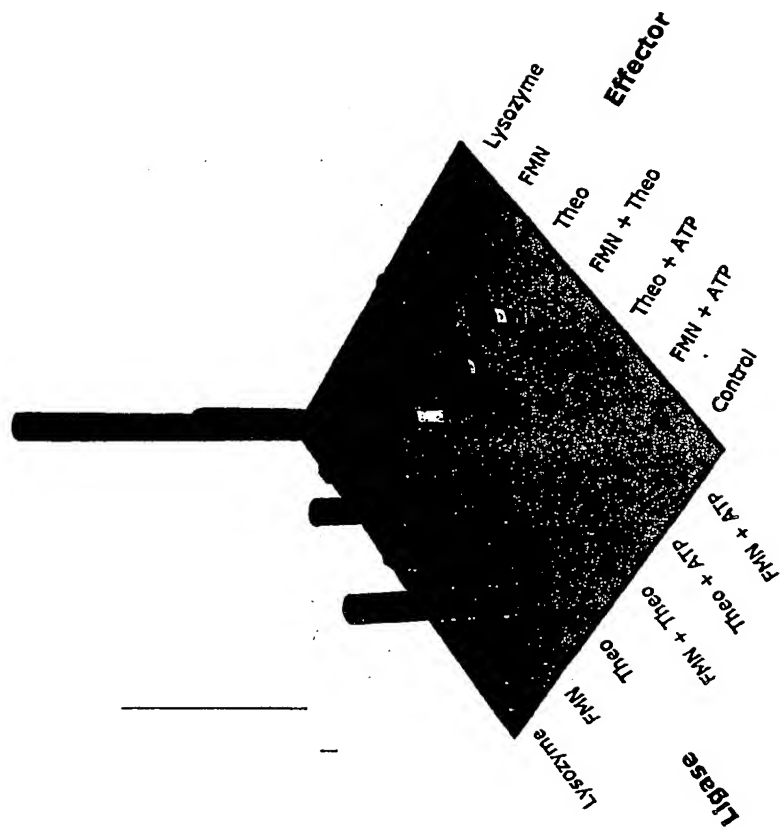
Figure 14.

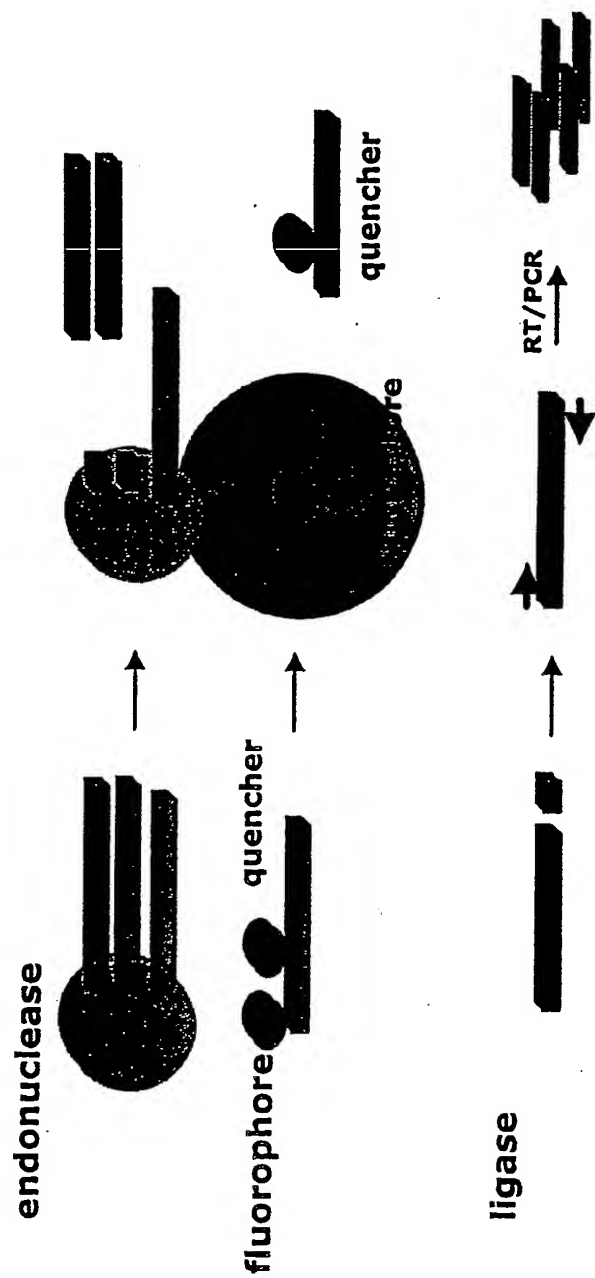RNA sensor SEQ ID NO:76

capture oligo SEQ ID NO: 77

FIGURE 15

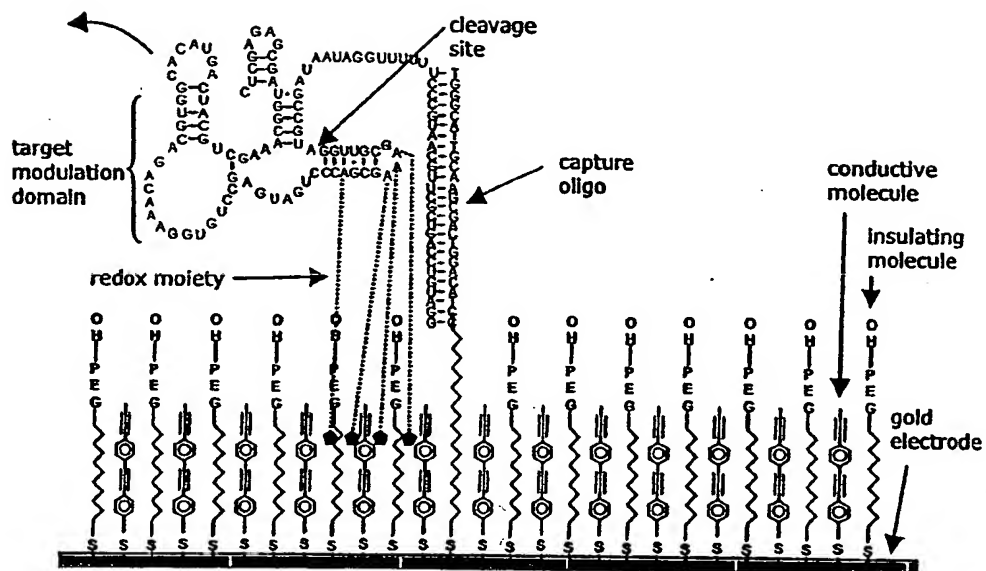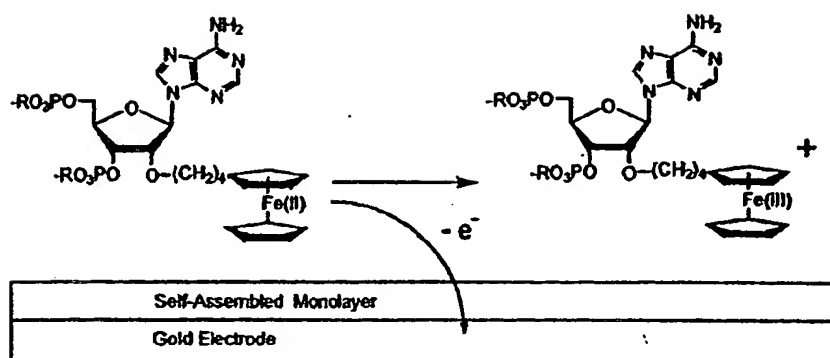RNA sensor SEQ ID NO:78
capture oligo SEQ ID NO:79

FIGURE 16

FIGURE 17
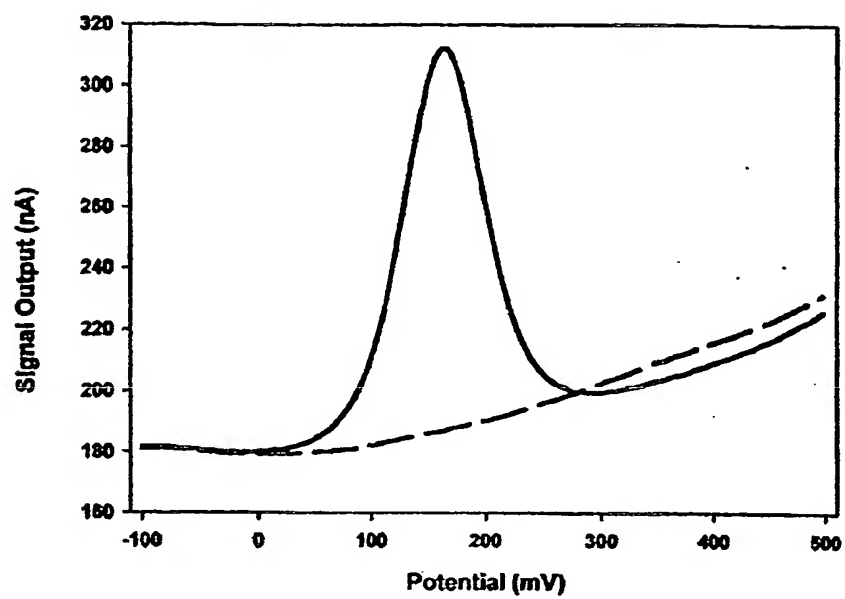
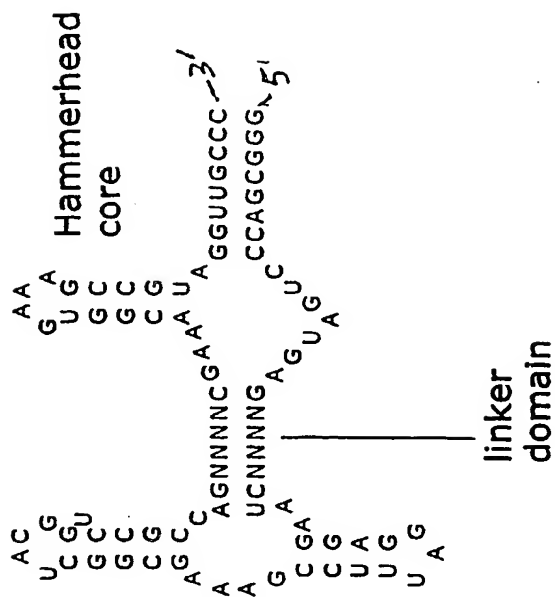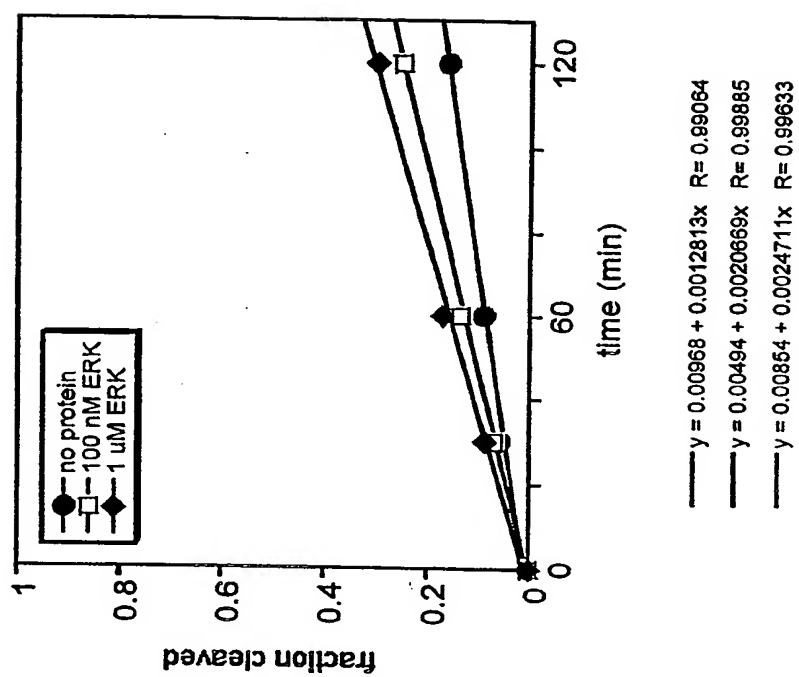FIGURE 18

SEQ ID NO:80

Target modulation domain

Hammerhead core

linker domain

Figure 19

	top -5′NNNN 3′ bottom-3′NNNN 5′	Activity
1-14	ACGU GCUGG	+++
1-13	UACGU UUCU	+++
1-2	ACGU UUCC	++
1-6	ACGU UAUC	+
2-7	CAGU UUCC	+++
2-2	CUAG AUCC	+
2-3	UUAA UCAA	+
2-13	CUGG AAAU	+
2-14	GAUC UUUU	+
2-20	UUAA AGCG	+

Figure 20A

Figure 20B

Figure 20C

Figure 21

Figure 22A

Figure 22B

SEQ ID NO: 353

Target Modulation Domain

Hammerhead core
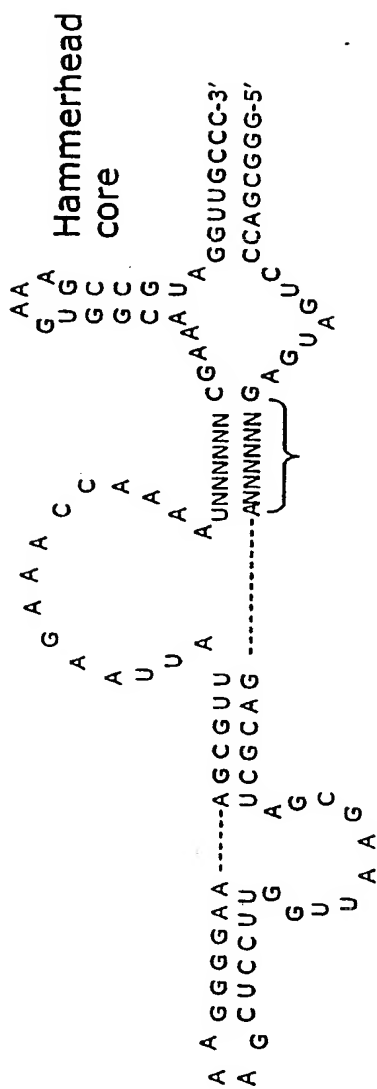
Figure 23A

Construct 6 SEQ ID NO: 81
GGGCGACCCUGAUGAGUCAGCGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUGUUACGAAACGGUGAAAGGCCGUAGGUUGCC
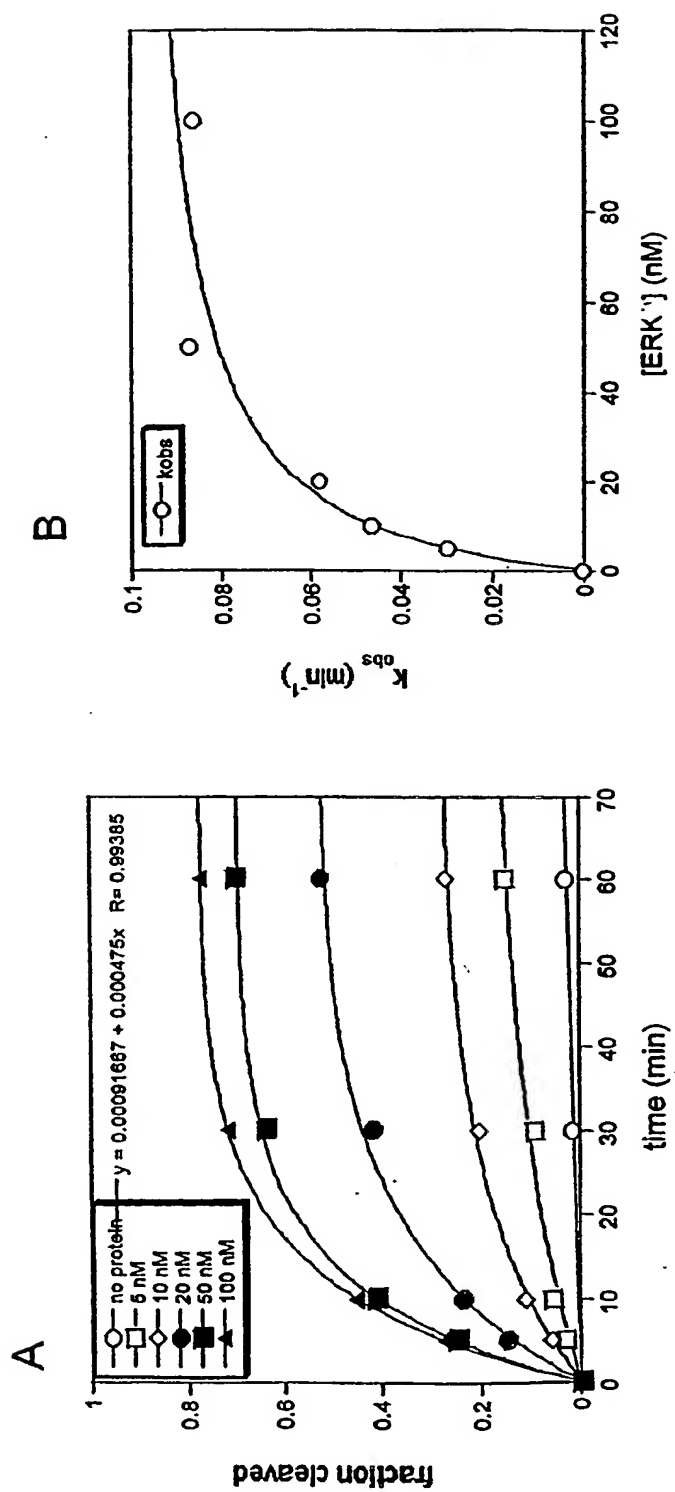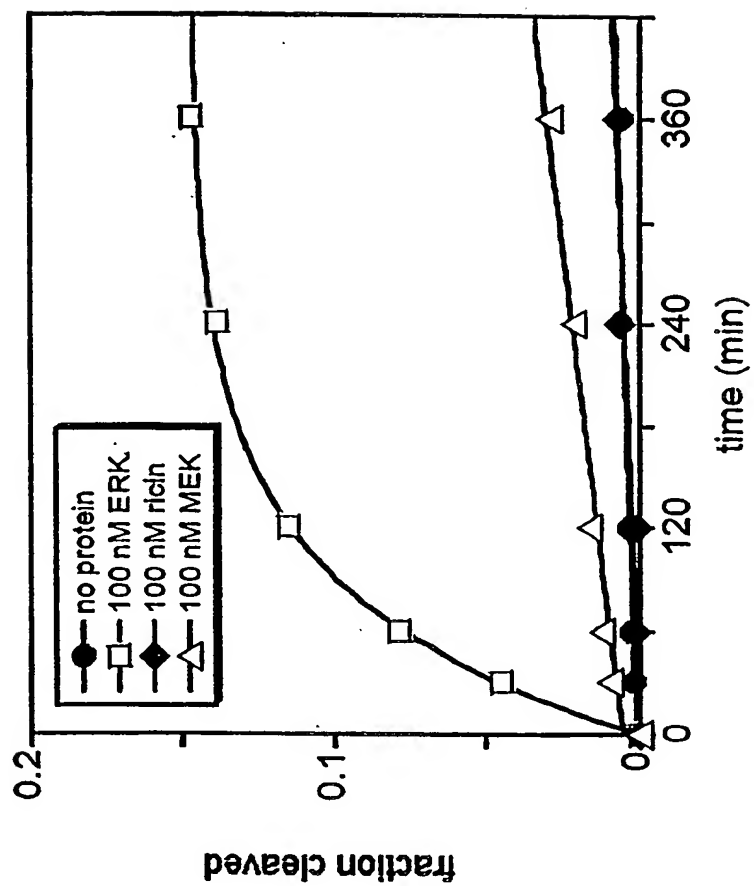
Construct 7 SEQ ID NO: 82
GGGCGACCCUGAUGAGAGUCACGAGACGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUGUUACGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 8 SEQ ID NO: 83
GGGCGACCCUGAUGAGAGUGAGACGCAGACGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUGUUCGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 9 SEQ ID NO: 84
GGGCGACCCUGAUGAGUCAGCGCAGACGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUUUACGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 10 SEQ ID NO: 85
GGGCGACCCUGAUGAGUCAGCGCAGACGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUGUUAUUAAGAAACCAAAAUGUGUUACGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 11 SEQ ID NO: 86
GGGCGACCCUGAUGAGAGUCACGCAGACGCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUGCGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 12 SEQ ID NO: 87
GGGCGACCCUGAUGAGAGCCUUGGCAGACGCUUGCAGACGCCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUCAAAAUGUCAAAAUGUGUUCGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 13 SEQ ID NO: 88
GGGCGACCCUGAUGAGUCUGGCAGACGCUGGCAGACGCCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUCAUACGAAACGGUGAAAGGCCGUAGGUUGCC

Construct 14 SEQ ID NO: 89
GGGCGACCCUGAUGAGUCUGGCAGACGCUGGCAGACGCCUAGCGAAUUGGGUUCCUCGAAUUGGGGGAAAGCGUUAUUAAGAAACCAAAAUGUCUUACGAAACGGUGAAAGGCCGUAGGUUGCC

Figure 23B

Figure 24

	sequences	activity	stability
Construct 6	GUGUUA CGCACU	++	-32.41Kcal
Construct 7	UGUUA GCACU	Constitutively active	-29.11Kcal
Construct 8	GUGUU CGCAC	Constitutively active	No predicted duplex formation
Construct 9	GUUUA CGACU	Constitutively active	-29.41Kcal
Construct 10	GUGUUG CGCACU	++	-32.7Kcal
Construct 11	GUG CGU	Constitutively active	-30.71Kcal
Construct 12	GUACGU CGUUCC	+++	No predicted duplex formation
Construct 13	GUCAUA CGGUCU	Constitutively active	-32.41Kcal
Construct 14	GUCUUA CGGUCU	Constitutively active	No predicted duplex formation

Figure 25

Figure 26

L1 ligase configured for
self-circularization

PCR

Original L1 ligase

Reverse
Transcribe

Circularized L1
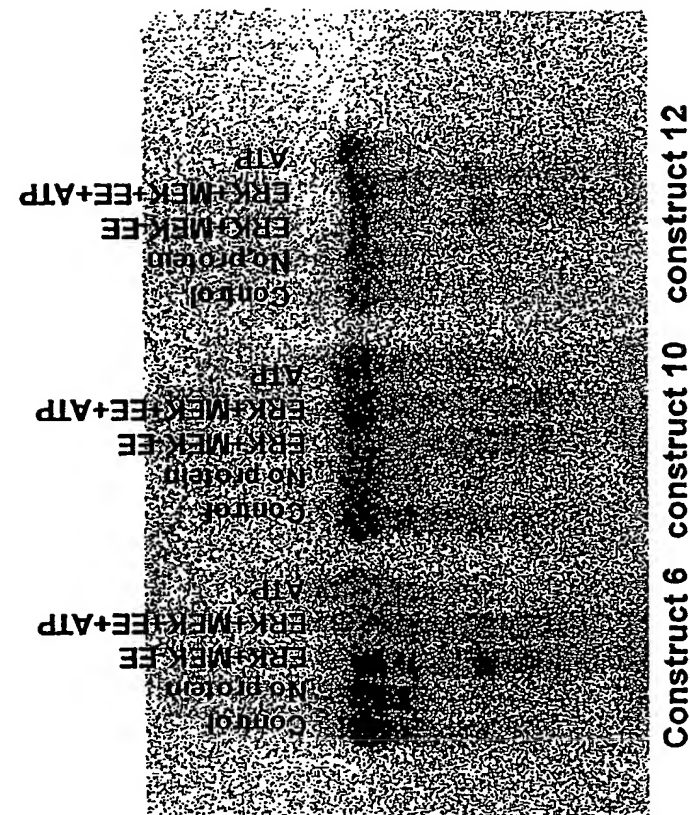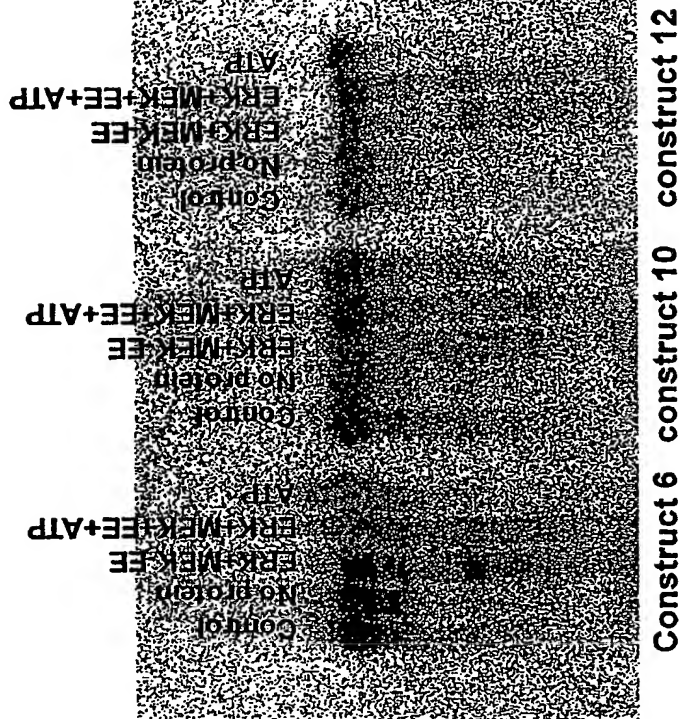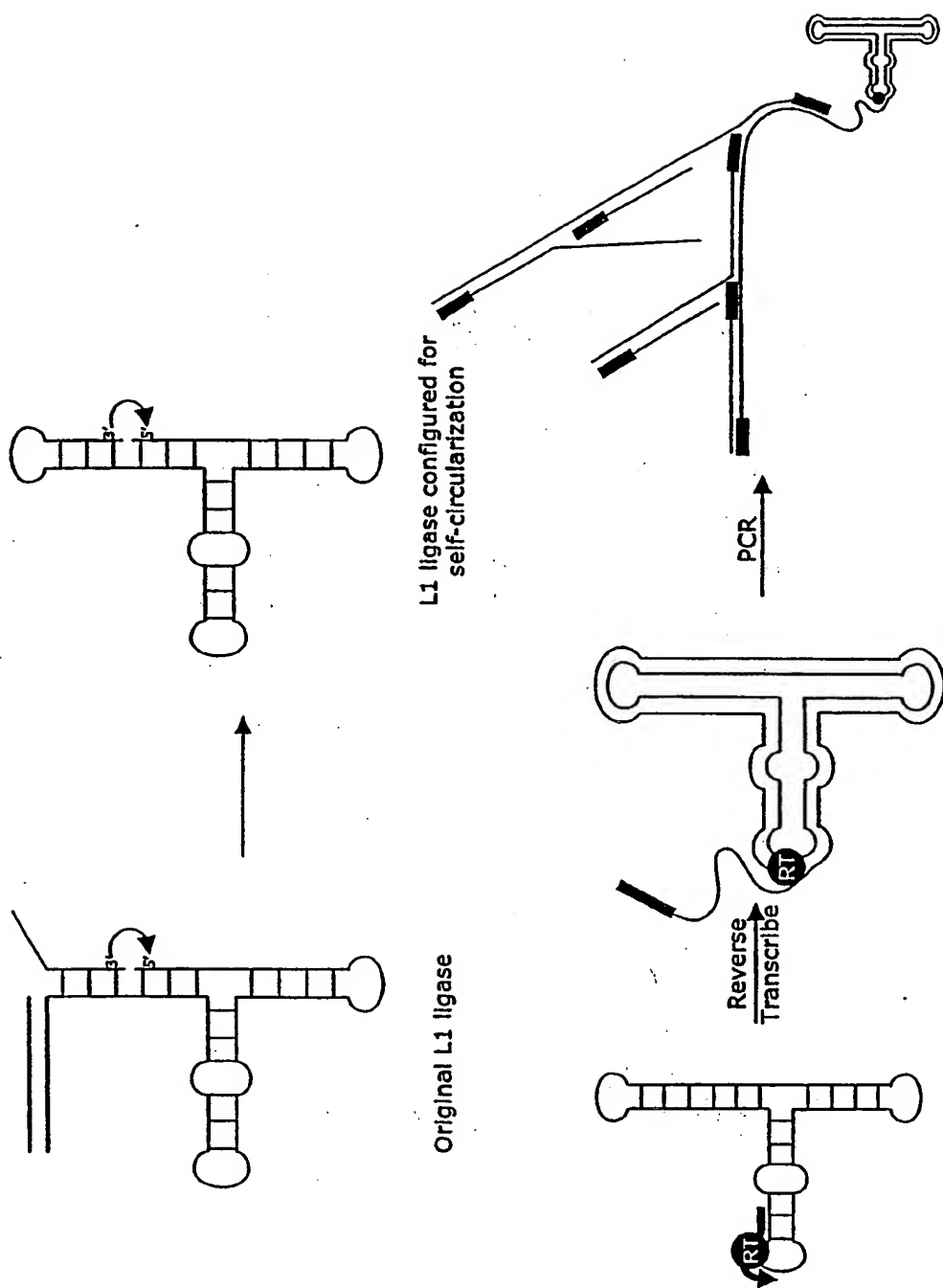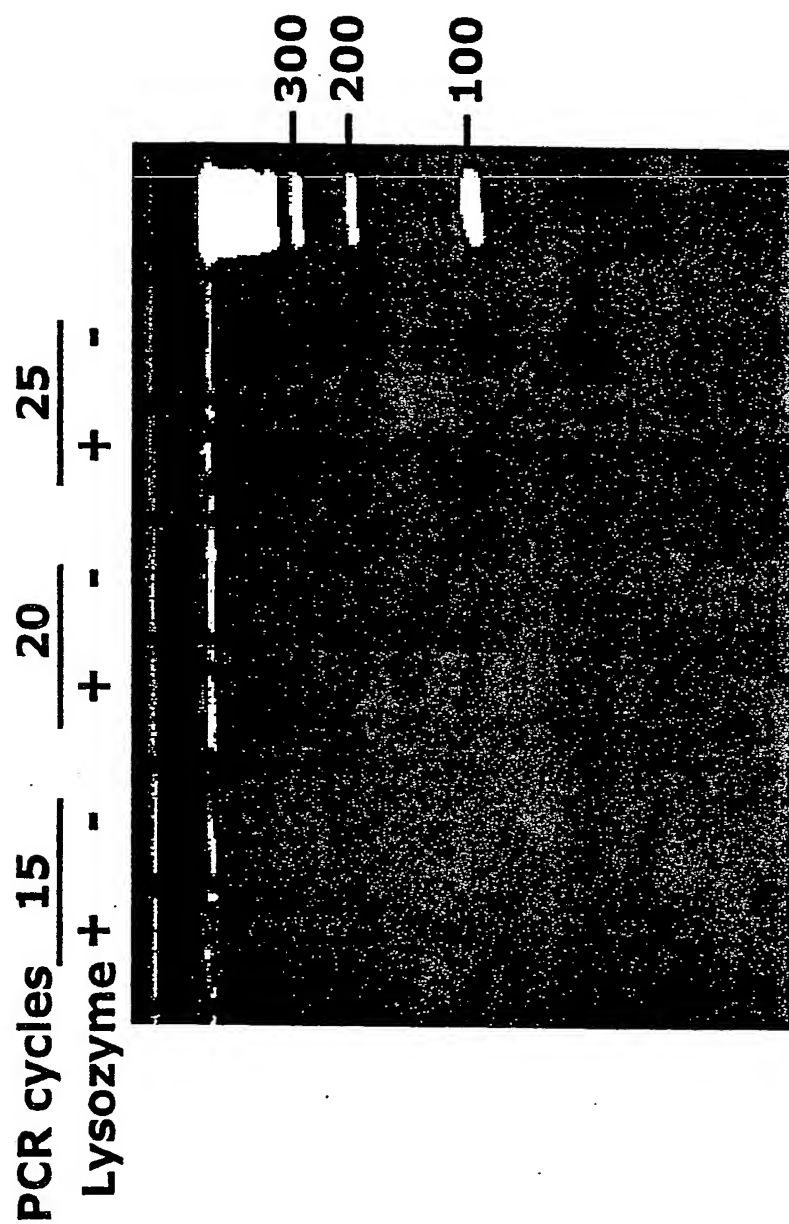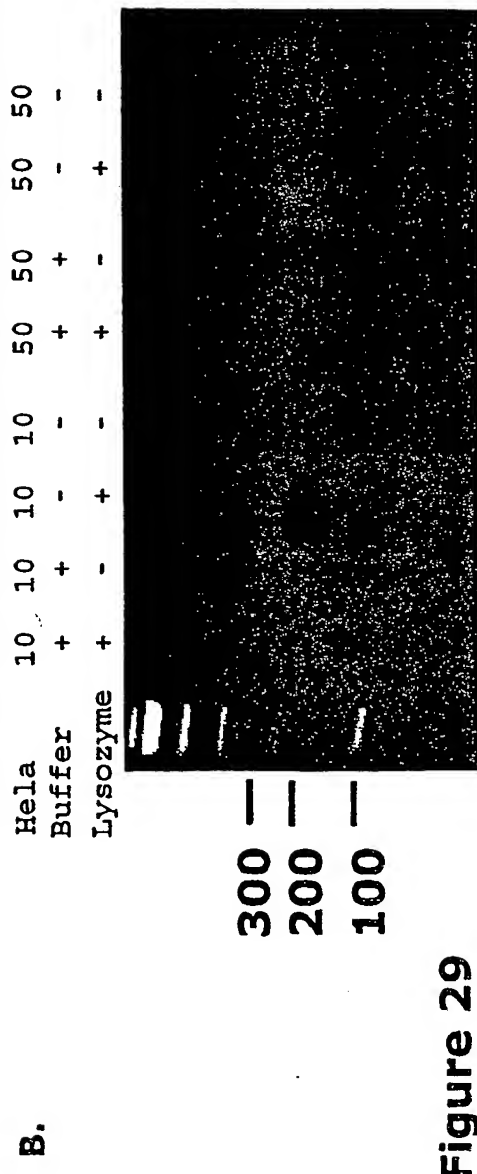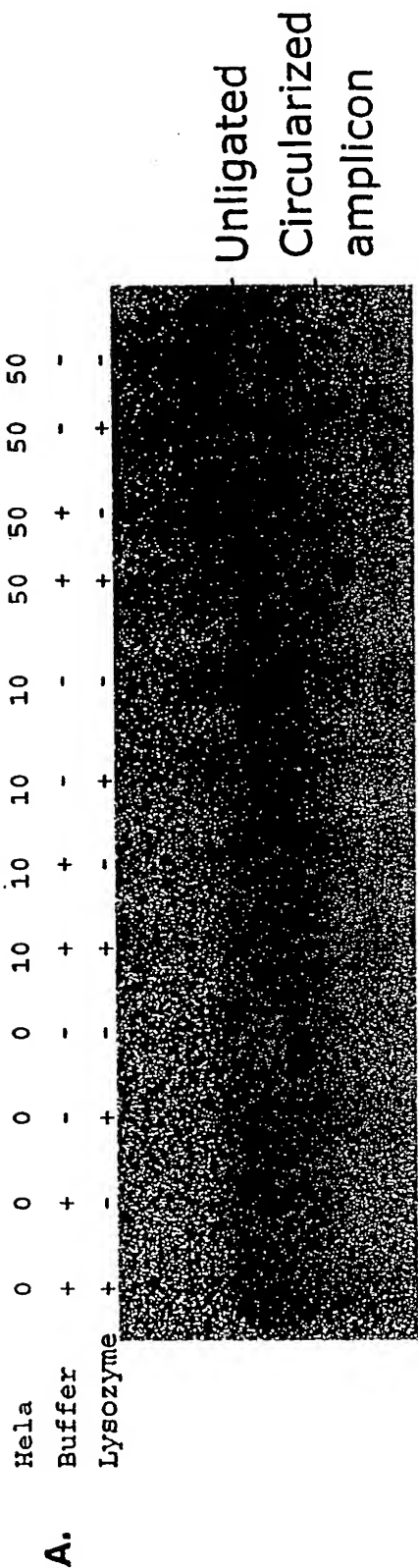
Figure 27

Figure 28

Figure 29

Figure 30
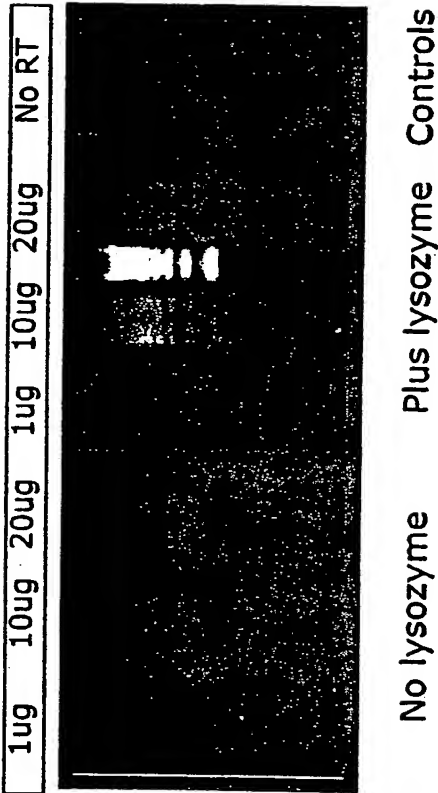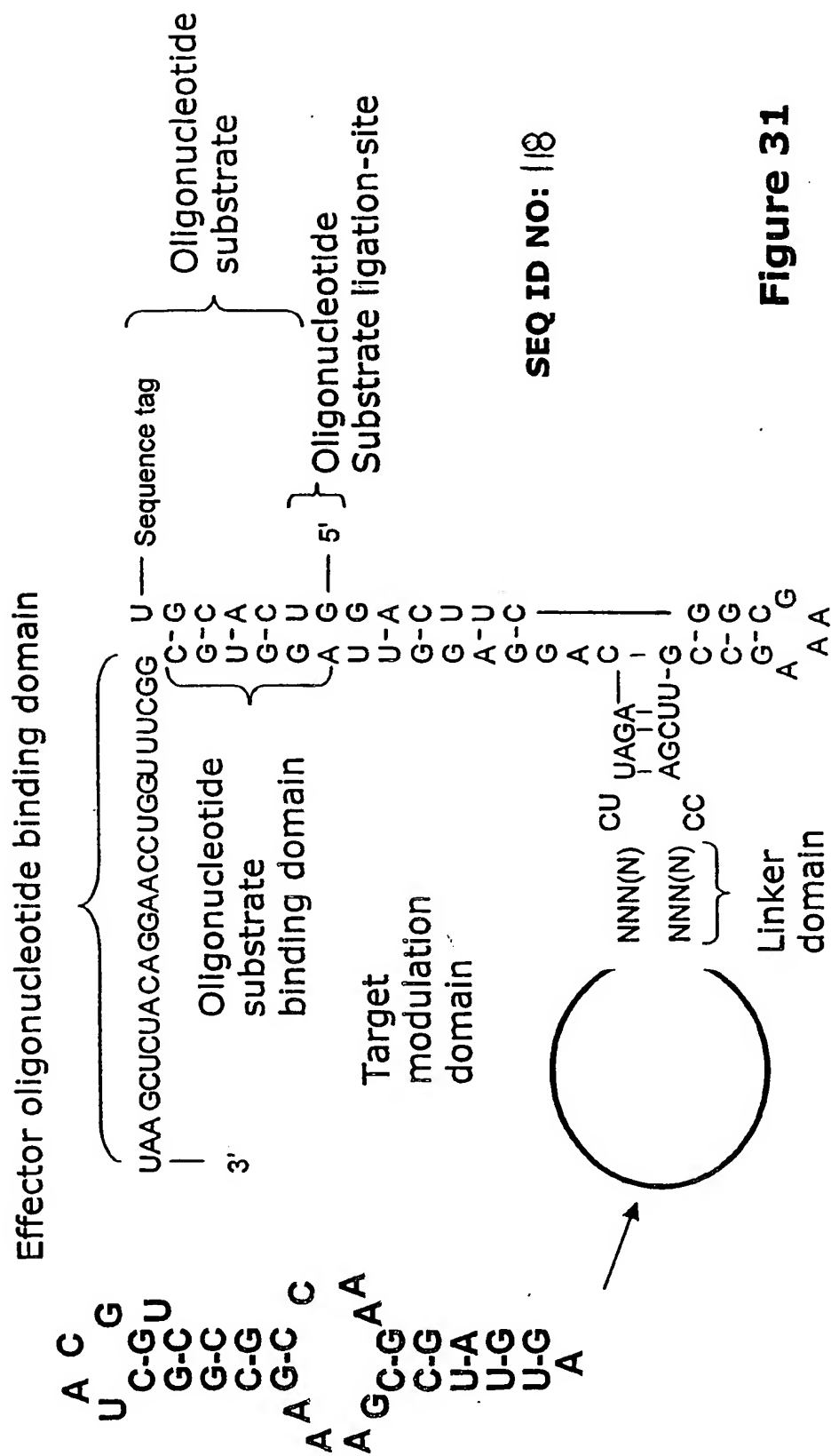
Figure 31

SEQ ID NO: 118

Sequences of ERK-modulated ligase nucleic acid sensor molecules

DG.20.58A   SEQ ID NO: 109   construct 17
GGACUUCGGGCGGAAAGCCGUUCGACCACGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUGUCUUAGACAG
GAGGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58B   SEQ ID NO: 110   construct 18
GGACUUCGGGCGGAAAGCCGUUCGACCAGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUUCUUAGACAGAGGA
GGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58C   SEQ ID NO: 112   construct 20
GGACUUCGGGCGGAAAGCCGUUCGACCACGGUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAUUGUCUUAGACAG
GAGGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58D   SEQ ID NO: 113   construct 21
GGACUUCGGGCGGAAAGCCGUUCGACCACCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCACGUUCUUAGACAG
GAGGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58E   SEQ ID NO: 111   construct 19
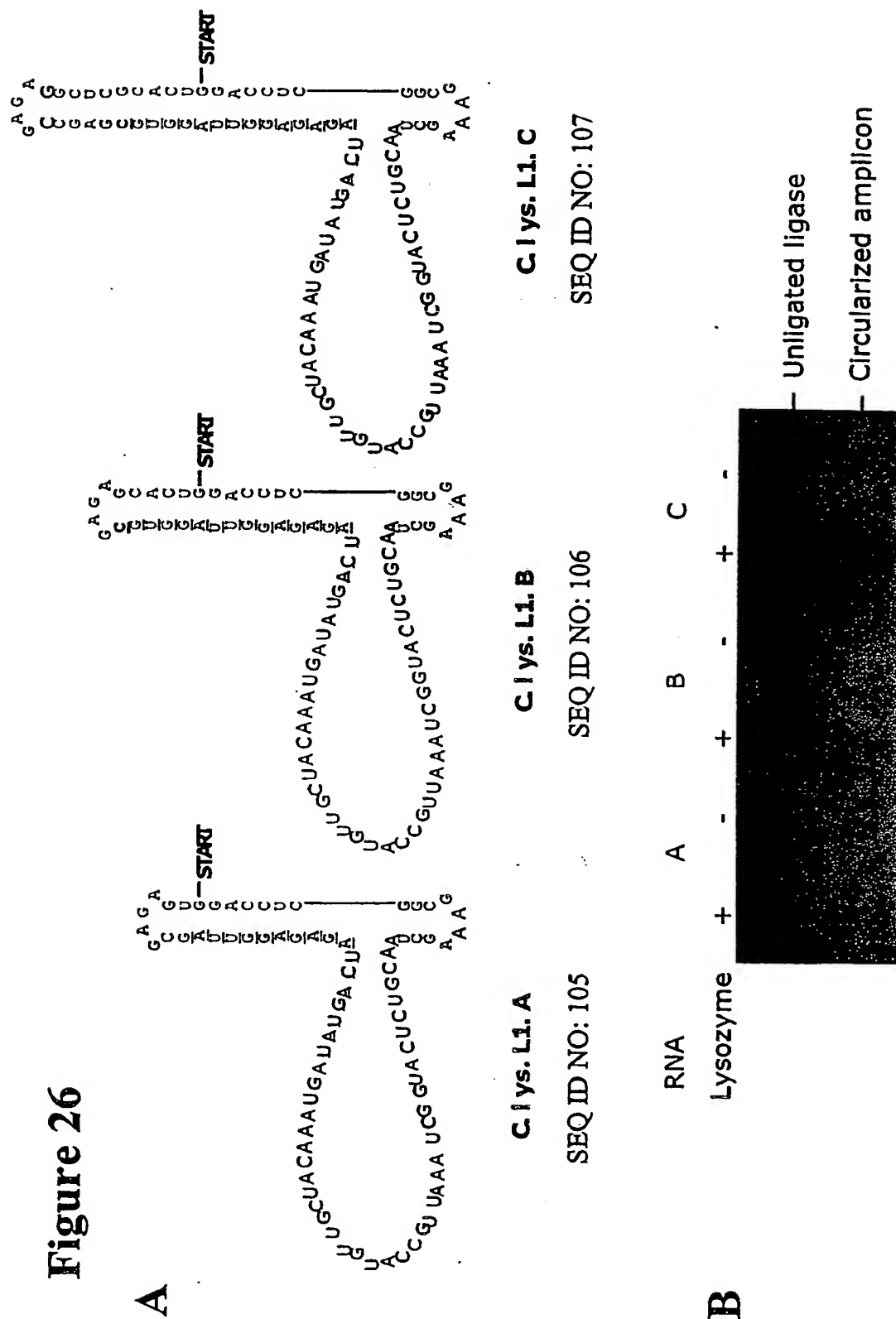GGACUUCGGGCGGAAAGCCGUUCGACCAGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGCUCUUAGACAGGA
GGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58F   SEQ ID NO: 114   construct 22
GGACUUCGGGCGGAAAGCCGUUCGACCGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUGCUUAGACAGGA
GGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58G   SEQ ID NO: 115   construct 23
GGACUUCGGGCGGAAAGCCGUUCGACCGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGUCUUAGACAGGAGG
UUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58H   SEQ ID NO: 117   construct 25
GGACUUCGGGCGGAAAGCCGUUCGACCGGUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAUUGCUUAGACAGGA
GGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58I   SEQ ID NO: 118   construct 26
GGACUUCGGGCGGAAAGCCGUUCGACCCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCACGUCUUAGACAGGA
GGUUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
DG.20.58J   SEQ ID NO: 116   construct 24
GGACUUCGGGCGGAAAGCCGUUCGACCGCUAAGGAGGAUUUCCGAAAGCGGCUACGGUCCGCCAGCUUUAGACAGGAGG
UUAGGGUGCCGUCCGACUGAUCUCGGAGUUAAACG
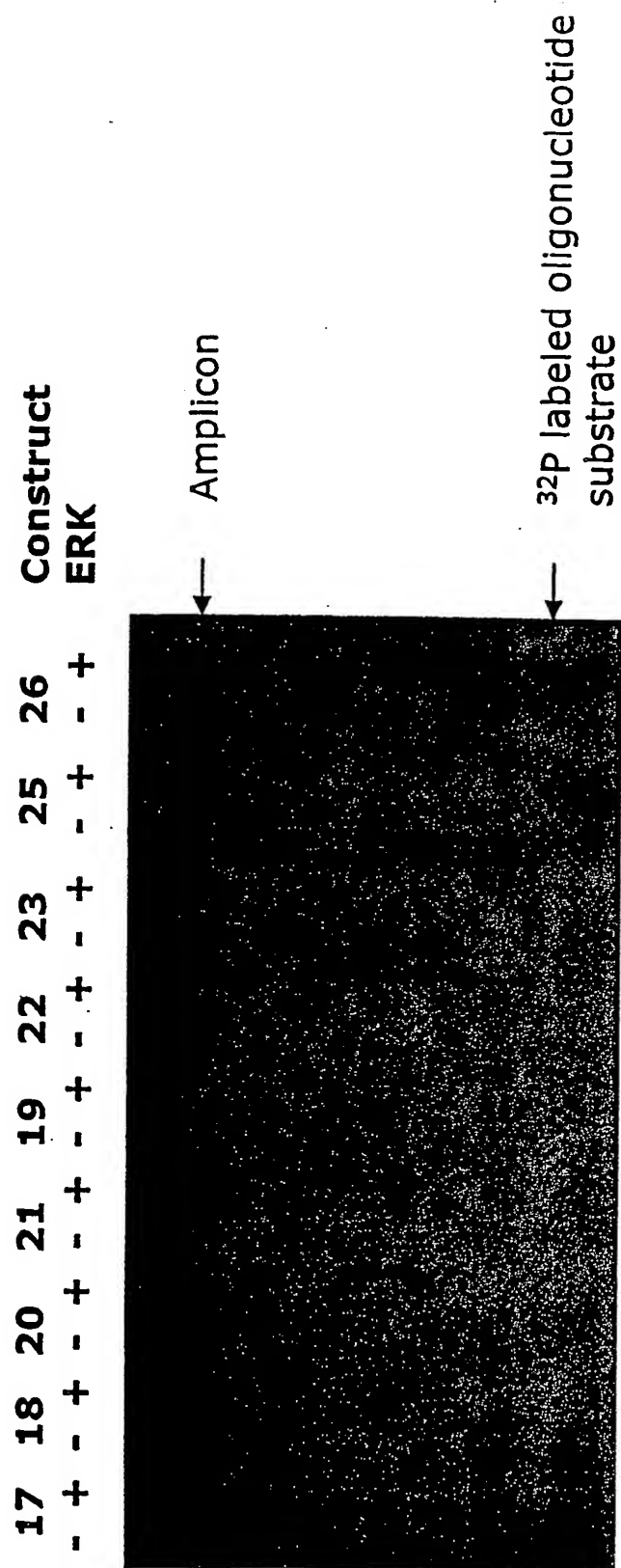
Figure 32

Figure 33

Figure 34
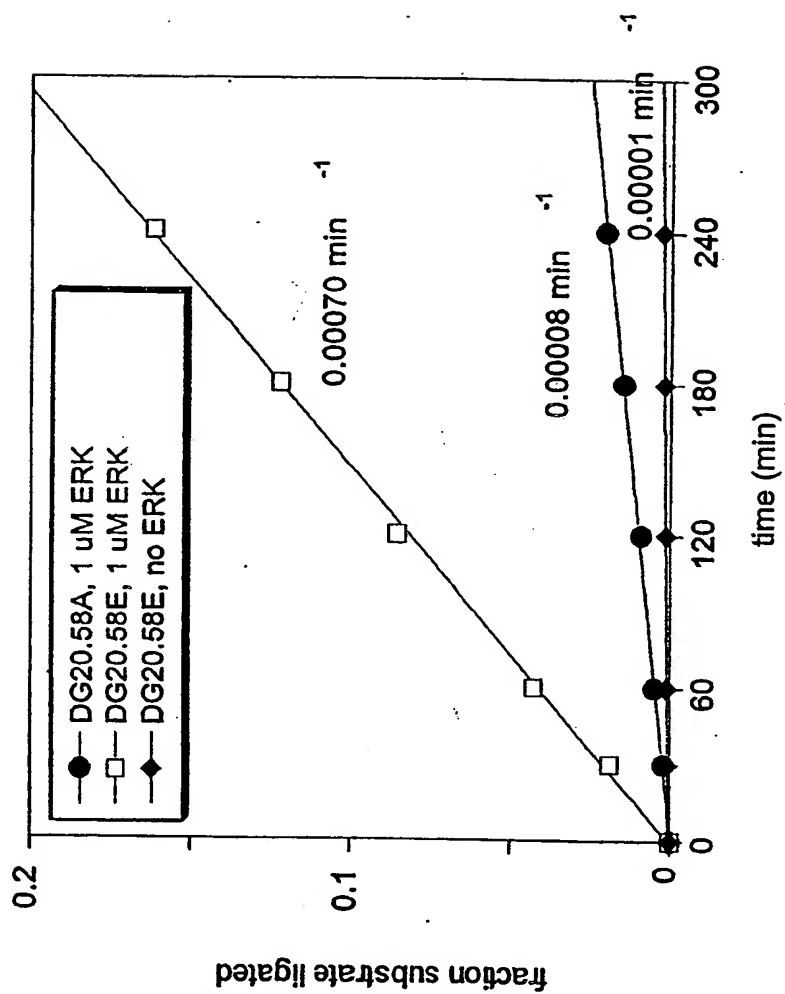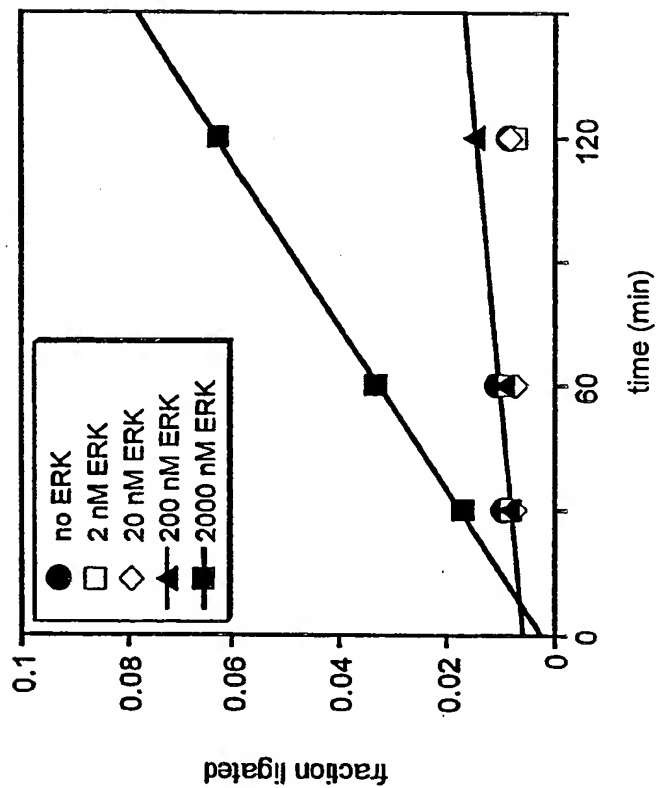
Figure 35

20 nM ERK: $k_{obs} = 8 \times 10^{-5}$ min^{-1}

200 nM ERK: $k_{obs} = 5 \times 10^{-4}$ min^{-1}

Figure 36

A

3-piece ligase platform

1-piece ligase platform

Construct 27
SEQ ID NO:108

Construct 28
SEQ ID NO:119

Figure 37

Figure 38

Construct 30

SEQ ID NO: 348

```
3' 5'
A·T
G-C
G-C
U-A
A·T          ← SEQ ID NO: 348
G-C
C-G
A·T
C-G
G-C
C-G
G-C
U-A
G-U 3'
A  G 5'
U  G
U-A
G-C
G  U
A-U
G-C ───────────
G      A   C  ─   C-G
              UAGA─  C-G
AGC  U  CU  ─    G·C
         ─  AGCUU-G    A
A  AGC-C C         A  A
UCG  A  CC
A  G-C-G   C-G
A  C-G     U-A
    C-G    U-G
A  C       U-G
U  G       A
   C-GU
   G-C
   G-C
   C-G
```

SEQ ID NO: 351

Construct 29

SEQ ID NO: 349

```
3' 5'
A·T
G-C
G-C
U-A
A·T          ← SEQ ID NO: 348
G-C
C-G
A·T
C-G
G-C
C-G
G-C
U-A
G-U 3'
A  G 5'
U  G
U-A
G-C
G  U
A-U
G-C ───────────
G      A   C  ─   C-G
              UAGA─  C-G
AGUG  U  CU  ─    G·C
          ─  AGCUU-G    A
A  AGC-C C         A  A
UCGC  A  CC
A  G-C-G   C-G
A  C-G     U-A
    C-G    U-G
A  C       U-G
U  G       A
   C-GU
   G-C
   G-C
   C-G
```

Figure 39A

Construct Number
ERK protein

Amplicon

29 30
- + -
- - +

32P labeled oligonucleotide substrate

Figure 39B

Figure 40

Effector-oligonucleotide-binding region

GCAAAUUGAGGCUCUAGUCAGCCUGC   U ── Sequence tag

C-G	
G-C	
U-A	**oligonucleotide substrate**
G-C	SEQ ID NO: 350
G-U	
A-G ── 5	
U-G	
U-A	
G-C	
G-U	
A-U	
G-C	
G	
A	
C ──	

3'

SEQ ID NO: 352

Target-modulation domain

Figure 41

Construct sequence Data

Constructs (these are RNA transcript sequences given as DNA sequences)

Construct 31 (TK.16.118.K) +   SEQ IDNO: 121
GGACTTCGGCGAAAGCCGTTCGACCACGCTAGCGACGCTAGGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATG
AGTGTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 32 (TK.16.118.L) +   SEQ IDNO: 122
GGACTTCGGCGAAAGCCGTTCGACCAGCTCAGACGCTAGGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGA
GTTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 33 (TK.16.118.M) ++   SEQ IDNO: 123
GGACTTCGGCGAAAGCCGTTCGACCACGGTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATG
ATTGTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 34 (TK.16.118.N) I   SEQ IDNO: 124
GGACTTCGGCGAAAGCCGTTCGACCACCTTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATG
ACGTTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 35 (TK.16.118.O) C   SEQ IDNO: 125
GGACTTCGGCGAAAGCCGTTCGACCAGCTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGA
GCTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 36 (TK.16.118.P) I   SEQ IDNO: 126
GGACTTCGGCGAAAGCCGTTCGACCCGCTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGA
GTGCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 37 (TK.16.118.Q) ++   SEQ IDNO: 127
GGACTTCGGCGAAAGCCGTTCGACCCGCTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGAG
TCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 38 (TK.16.118.R) ++   SEQ IDNO: 128
GGACTTCGGCGAAAGCCGTTCGACCCGGTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGA
TTGCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 39 (TK.16.118.S) I   SEQ IDNO: 129
GGACTTCGGCGAAAGCCGTTCGACCCCTTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGA
CGTCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

Construct 40 (TK.16.118.T) +   SEQ IDNO: 130
GGACTTCGGCGAAAGCCGTTCGACCGCTCAGACGCTAGCGAATTGGTTCCTCGAAAGGGGAAAGCGTTATTAAGAAACCAAAATGAG
CCTTAGACAGGAGGTTAGGTGCGGCTTTGGTCCAAGGACATCTCGAAT

I   Inactive
C   Constitutively Active
+   Effector-dependent
++   Greater Effector-Dependence than +

Figure 42

Figure 43

- ppERK library

5'GGACTTCGGGCGAAAGCCCGTTCGACCNNNNN(N)(N)CAGACGCTA
GCGAATTGGTTCCTCGAAAGGGAAAGCGTTATTAAGAAACCAAAA
TGNNNNN(N)(N)CTTAGACAGGAGGTTAGGTGCCGTCAATGCTGCAA
GTTACTG 3' SEQ ID NO: 354

- ERK library

GGACTTCGGGCGAAAGCCCGTTCGACCNNNN(N)(N)AAGGAGGATTTC
CGAAAGCGGCTACGGTCCGCCNNNN(N)(N)CTTAGACAGGAGGTTA
GGTGCGTAGGTAACCGATAGTTCCG SEQ ID NO:355

Figure 44A

SEQ ID NO:356

```
                G
                A
AGAGGcu  uAGA —— C
      UCUC  cc  AGCUU-G  C-G
                         U-G
```

CW45-33-A08 (3.18, 2/18)

AGGGG3'
UCUCC 5'

CW45-33-C08 (11.3, 1/18)

SEQ ID NO:131

AACAG
UAGU

CW45-33-C09 (18.6, 1/18)

SEQ ID NO:132

AGUAG
UCAU

CW45-33-F08 (9.5, 1/18)

SEQ ID NO:90

ACAAG
UGUCC

CW45-33-H08 (36, 1/18)

SEQ ID NO:91

AGCGA
UCGCU

CW45-33-H09 (33, 1/18)

SEQ ID NO:92

GGUUG
UCAA

CW45-33-A10 (11.4, 1/18)

SEQ ID NO:93

AACAG
UUGU

CW45-33-F09 (14.2, 2/18)

SEQ ID NO:94

AGAAG
UCUUC

CW45-33-G08 (11.4, 1/18)

SEQ ID NO:95

AAAAG
UUUU

CW45-33-D09 (17.3, 5/18)

SEQ ID NO:133

Designed Library:
3-5 nt stem
25% of each A, C, U, G
37.5% complementary

Result of selection:
4-5 nt stem
27.8% A
27.8% U
25.6% G
18.9% C
1st pair 90% A-U
2nd pair 60% GC
95.5% complementary

Figure 44B

Designed Library:
4-6 nt stem
25% of each A, C, U, G
37.5% complementary

Result of selection:
4-6 nt stem
4-6 pairing
18.0% A
28.9% U
36.7% G
16.4% C

89.8% complementary

```
GAACCGCU
          A
        UAGA  C
              -
        AGCUU-G
GUUGG CC      C-G
              T_G
```

CW45-33-A02 (4.46, 1/31)
One C->U mutation in 5' ligase
SEQ ID NO:44

UGAUCG3'
GUUGG5'
CW45-33-B04(5.05, 5/31)
SEQ ID NO:45

CGUGU
GUACG
CW45-33-C04(6.2, 1/31)
SEQ ID NO:5

UUAUCG
GAUGG
CW45-33-D04(9.0, 1/31)
One deletion, one A to G
mutation in aptamer structure
SEQ ID NO:6

CGCAGG
GGUGUC
CW45-33-F03(4, 1/31)
SEQ ID NO:7

CW45-33-D01(2, 1/31)
AGGG
UCAUC
SEQ ID NO:37

CW45-33-D02(3, 1/31)
AGUAAG   SEQ ID
UCUU   NO:38
CW45-33-D05(2, 1/31)
CGGUGG   SEQ ID
GACGC   NO:39
CW45-33-E01(2, 1/31)
GCAGAU   SEQ ID
UGUUUA   NO:96
CW45-33-G02(3, 1/31)
AUUAGA   SEQ ID
UGAUUU   NO:97
CW45-33-G03(3, 1/31)
AUCGG   SEQ ID
UCGC   NO:98
CW45-33-H03(2, 1/31)

GUGUGG   SEQ ID
AACGC   NO:8
CW45-33-D01(2, 1/31)
CAAU   SEQ ID
GUUG   NO:99
CW45-33-H1(1, 3/31)
CUAA   SEQ ID
GAUU   NO:100
CW45-33-B05(1, 1/31)

Figure 45

● CW45.33.A02 sequence SEQ ID NO:44

5'**GGACUUCGGGGCGAAAGU**☐**UCGUUCGACC**GGUUGCAGACGCUAGCGA
AUUGGUUCCUCGAAAGGGAAGGGGAAAGCGUUAUUAAGAAACCAAAAUGGAA
CCGCUUAGAGACAGGAGGGUUAGGGUGC3'

● CW45.33.D04 SEQ ID NO:6

5'**GGACUUCGGGGCGAAAGCCGUUCGACC**GGUAGCAGACGCUAGCGA
AUUGGU_CCUCGAA☐**G**GGGGAAAGCGUUAUUAAGAAACCAAAAUGUUA
UCGCUUAGAGACAGGAGGGUUAGGGUGC3'
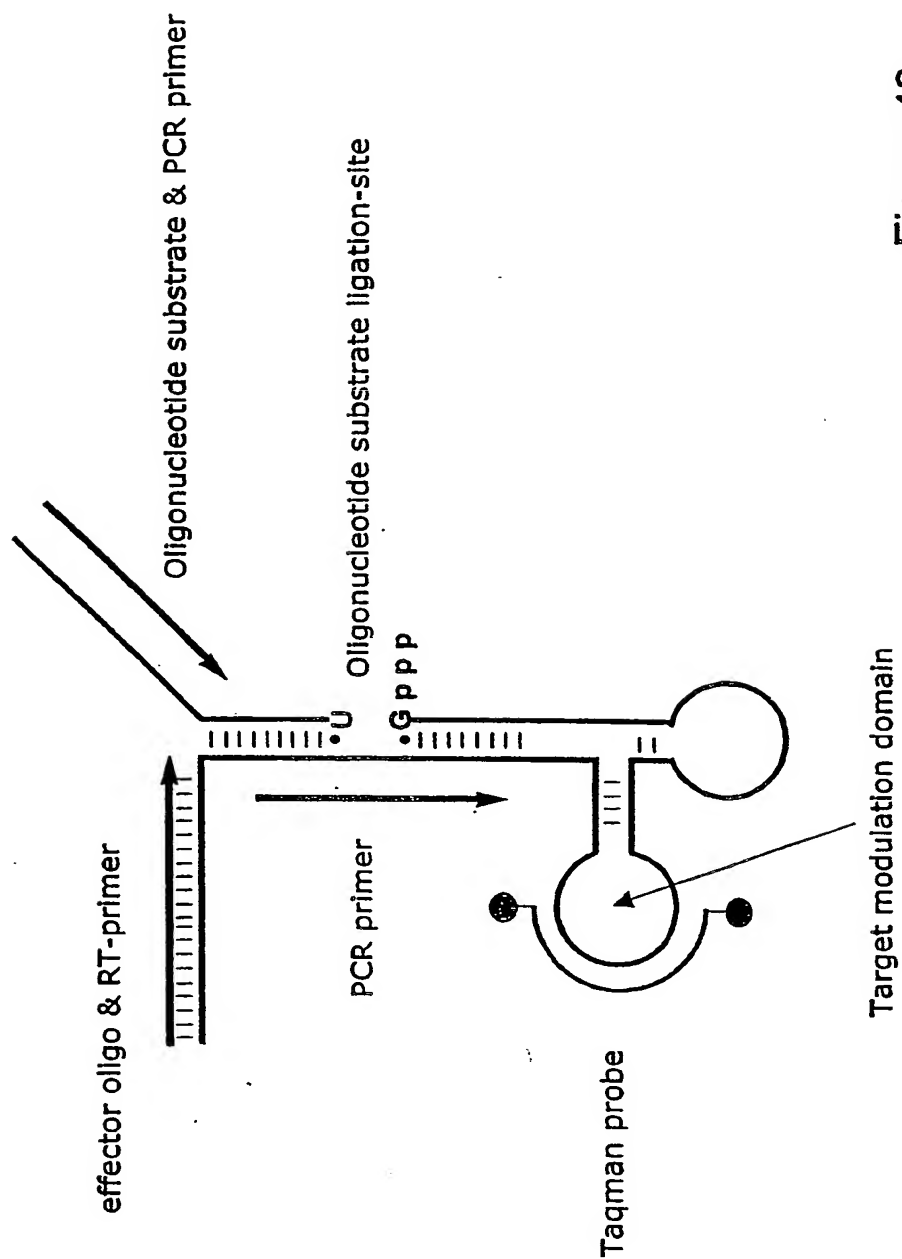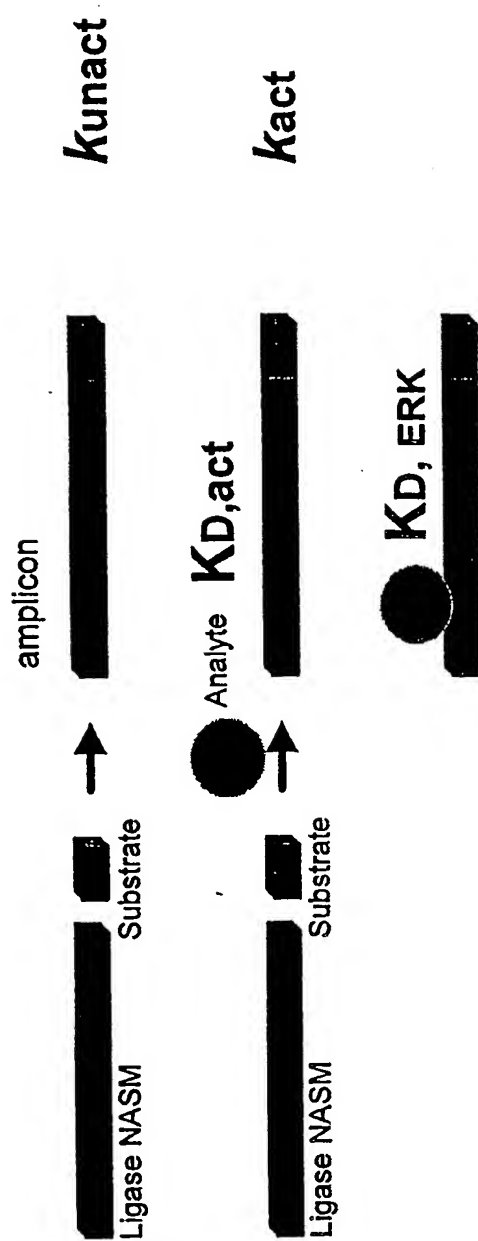
Figure 46

Figure 47

Switch factor = *kact/ kunact*

Figure 48
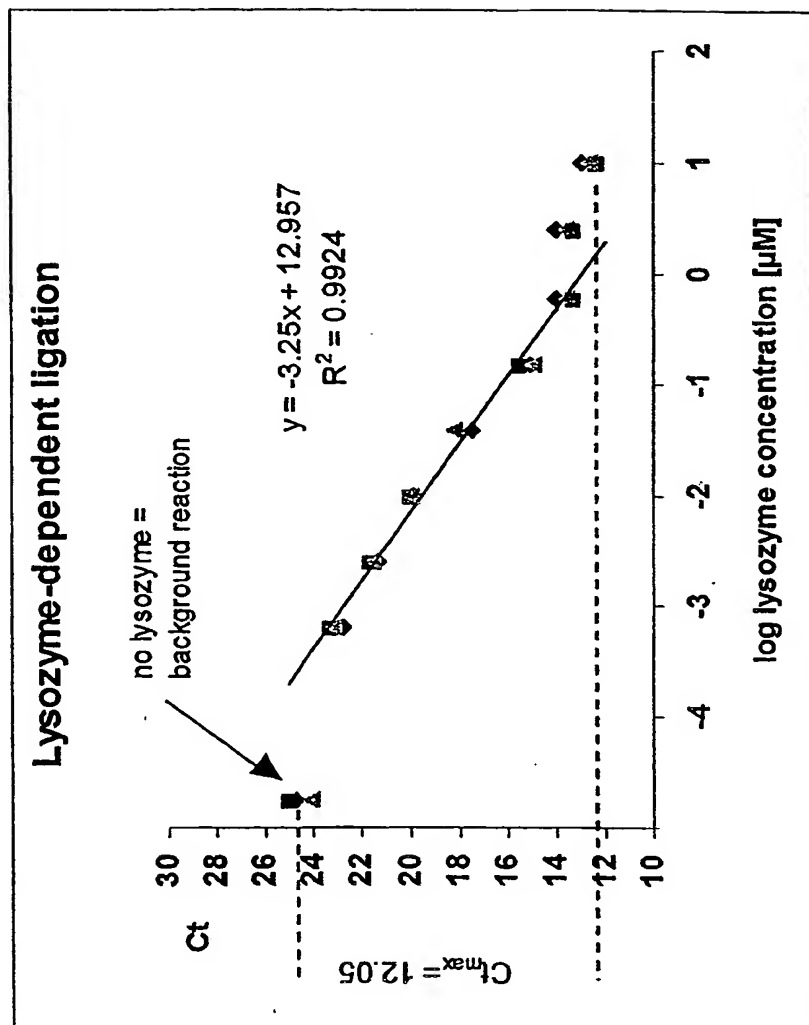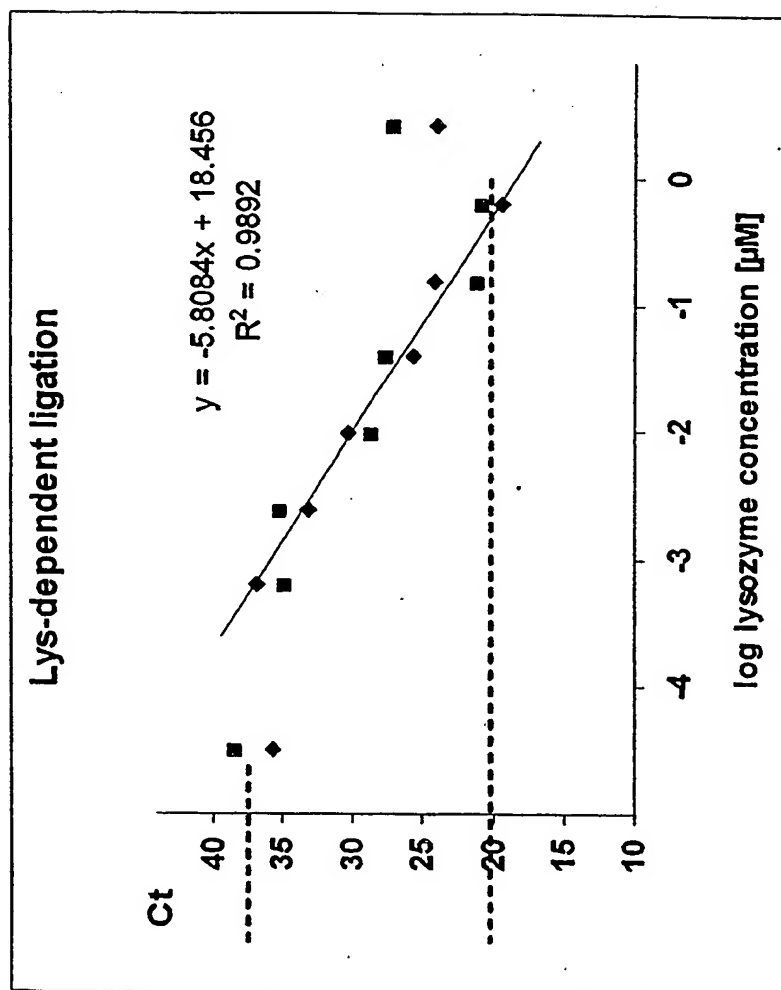
Figure 49
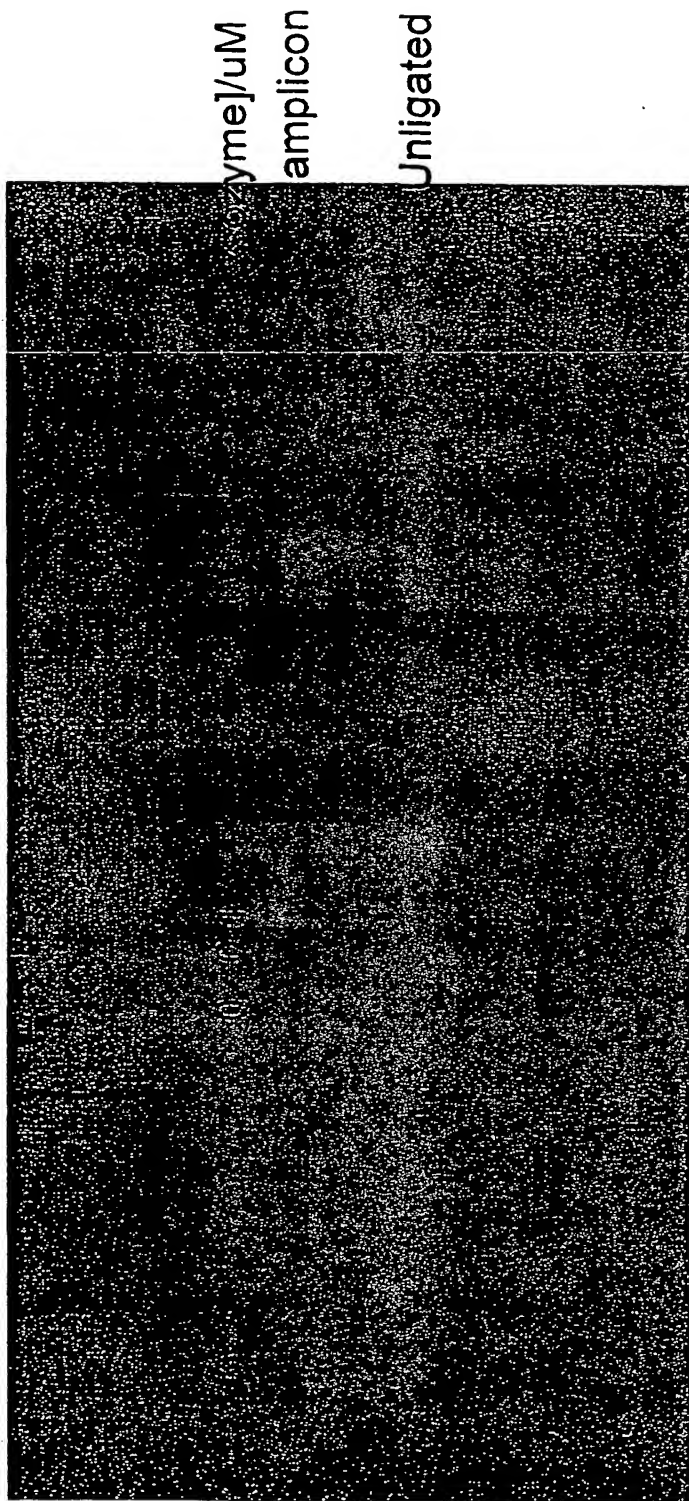
Figure 50

Figure 51

Figure 52

Target modulation domain

target

ligase drives circularization of a linear RNA substrate in the presence of the target

The 3'-end of the ribozyme serves as a primer to initiate reverse transcriptase catalyzed extension of the circularized substrate.

The 5'-end of the ribozyme is attached to a solid support via a 5'-end modification (*e.g.* thiophosphate)

Labeled NTPs (*) can be incorporated during rolling circle replication to generate a detectable signal

Figure 53

1. polymerase catalyzes rolling circle synthesis

2. primer A hybridizes with rolling circle concatemer, initiates complement synthesis

3. primer B hybridizes with complement, initiates 2nd strand synthesis
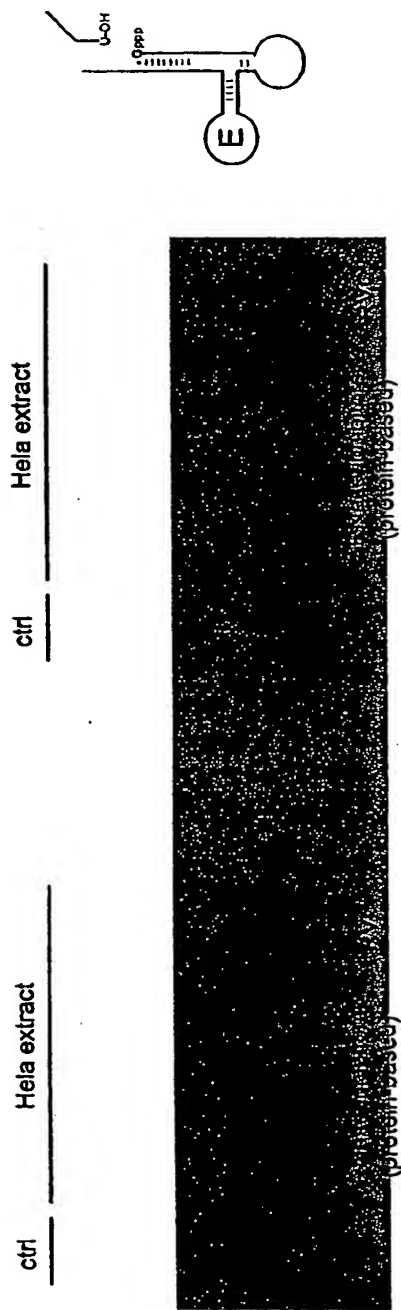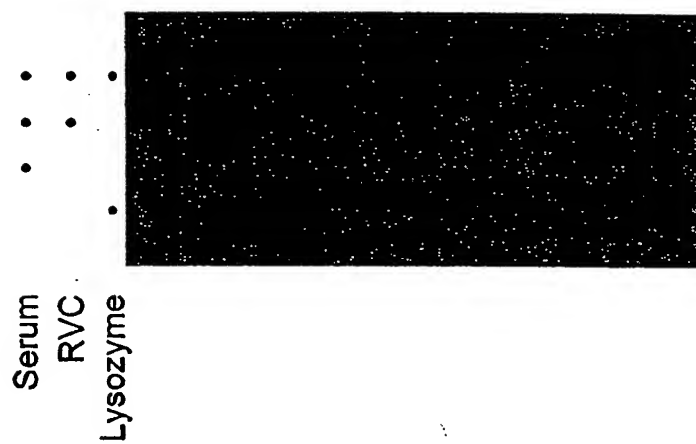
Figure 54

Figure 55

Figure 56

transfect

RT-PCR

- Intracellular target
  drives circularization of
  transfected nucleic acid
  sensor molecule

- RT-PCR amplifies
  circular form to generate
  signal

Panel B: Specificity for MAP kinase component
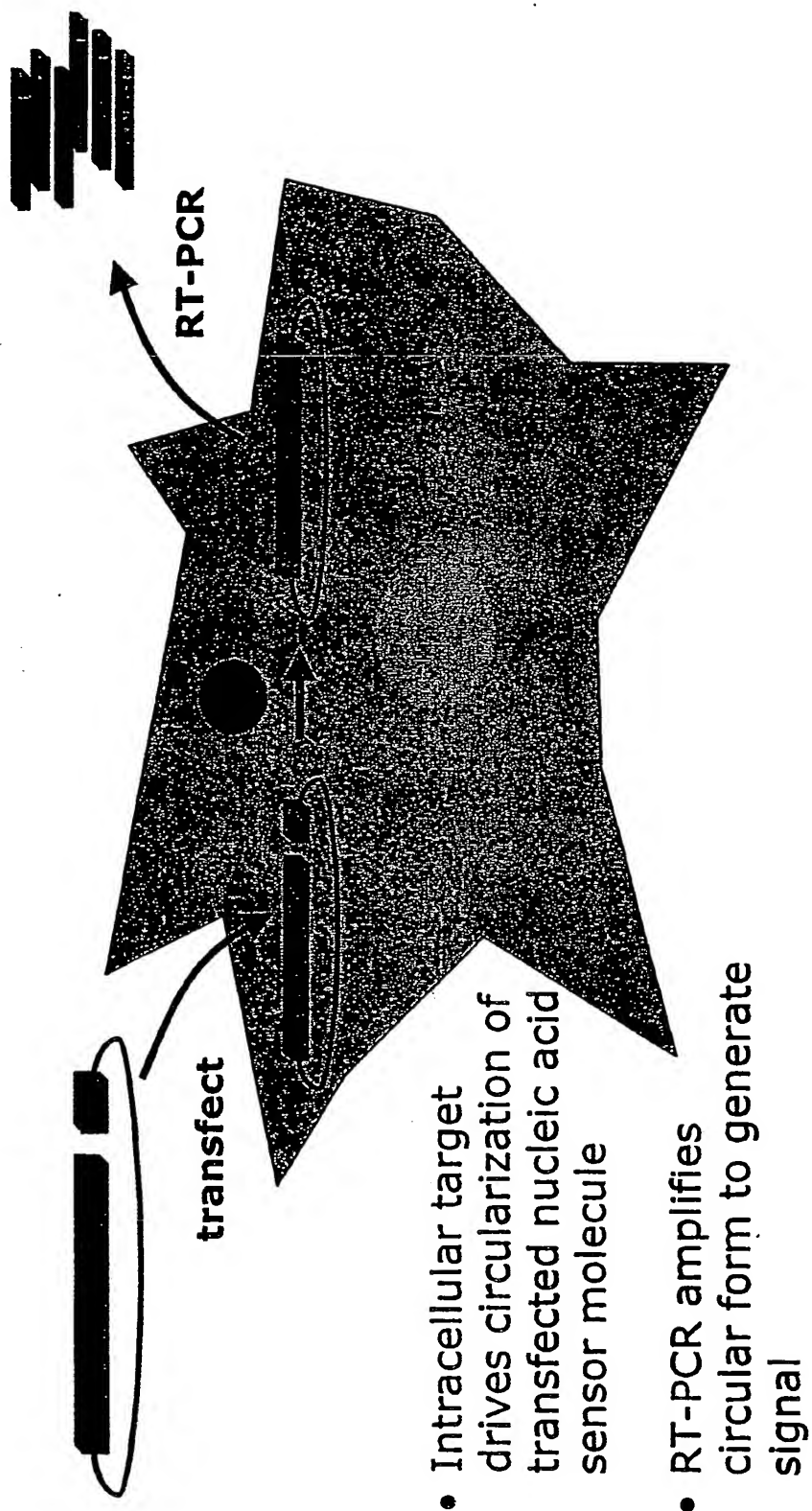
Panel A: Specificity for phosphorylation
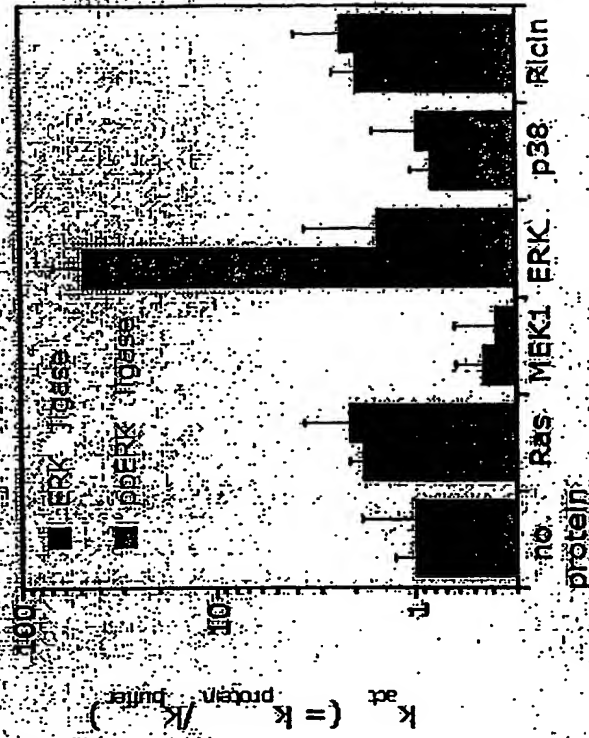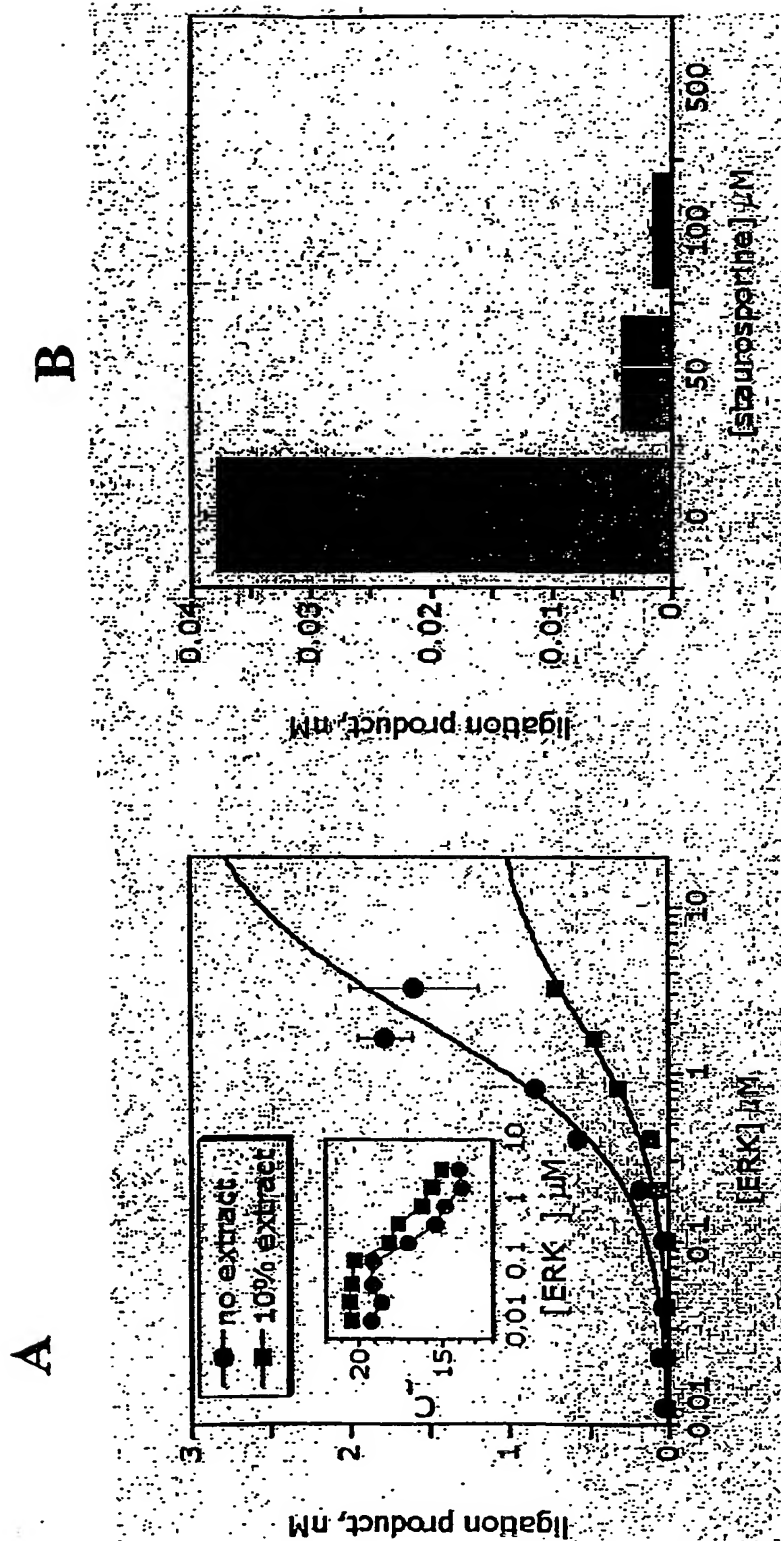
Figure 57,

Figure 58

Figure 59

ERK sensor	Switch factor	$K_{D,act}$ µM	$K_{D,ERK}$ µM	k_{unact} nM/min	Det. limit
ERK aptamer	N/A	N/A	0.0043 ± 0.0005	N/A	N/A
Ligase 1	≥800	≥5	≥5	~0.004	40 nM
Ligase 2	76 ±20	0.75 ±0.08	—	0.04 ±0.01	10 nM
Ligase 3	65 ±16	0.28 ±0.08	1.2 ±0.1	~0.02	10 nM
Ligase 4	5 ±1	0.04 ±0.02	0.17 ±0.07	~0.3	40 nM

Figure 60

C. ADNA by Taqman®

D.

A. ADNA by RT-PCR

B.

Figure 61

A

λ_{EX}

RNA sensor SEQ ID NO:101 (cGMP)
quencher oligo SEQ ID NO:102

```
    U G A
   C     C
  G       G
   U—A
   G—C
   G       A
  A        C
 A          A
A A         U    G A A
                G  U-G  A
                   G-C
                   G-C
                   C-G
                   A-U   A GGUCU    TGGGCATTGCAAGCGACTGGACATCC
                              ||||||||||||||||||||||||||
                              CCCAGAUUUUCCCGUAACGUUCGCUGACCUGUAGG 5'
```

B

RNA sensor SEQ ID NO: 101 (cGMP)
capt. oligo + biotin SEQ ID NO: 102

λ_{EM} λ_{EX}

```
    U G A
   C     C
  G       G
   U—A
   G—C
   G       A
  A        C
 A          A
A A         U    G A A
                G  U-G  A
                   G-C
                   G-C
                   C-G
                   A-U   A GGUCU    TGGGCATTGCAAGCGACTGGACATCC-Biotin—SA
                              ||||||||||||||||||||||||||
                              CCCAGAUUUUCCCGUAACGUUCGCUGACCUGUAGG 5
```

FIGURE 62

A.

λ_EM   λ_EX   RNA sensor SEQ ID NO:101 (cGMP)
                capt. oligo + biotin SEQ ID NO: 102

B.

cGMP

RNA sensor SEQ ID NO: 101 (cGMP)
capture oligo + biotin SEQ ID NO: 102

cGMP

C.

Expected time-course :

FI

time

D.

FRET donor signal (FAM)

0 uM
100 uM
600 uM

time (min)

FIGURE 63

A.

solution-phase FRET: cGMP HH (FAM -> TAMRA)
200 uM cGMP, 96 well, black Proxi-plate: 10 uL/well
replicate 1

B.

Solution-phase FRET: cGMP HH (FAM->TAMRA)
10 uL/well, 200 uM cGMP, 3.22.02 data

$$y = m1*(1 - \exp(-1*M0*m2))$$

	Value	Error
m1	0.95085	0.011671
m2	0.37796	0.018537
Chisq	0.013245	NA
R^2	0.98776	NA

FIGURE 64.

FIGURE 65A and 65B
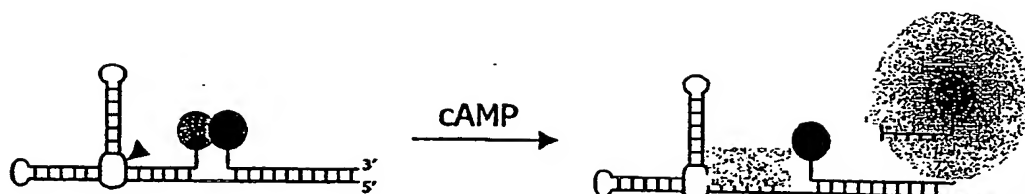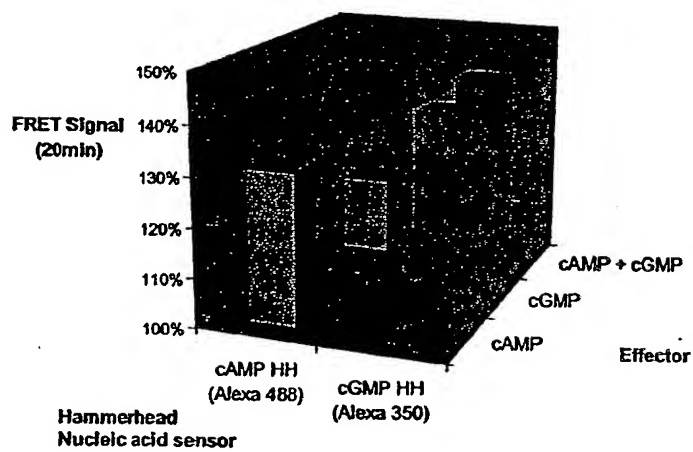
FIGURE 65C, 65D and 65E

A.
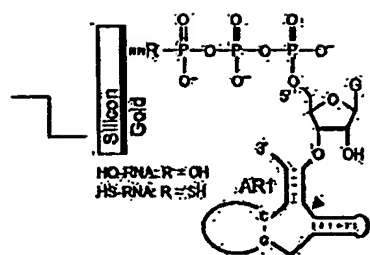
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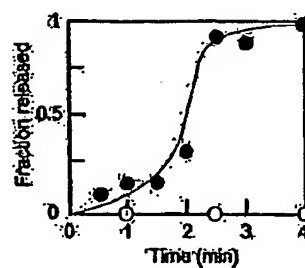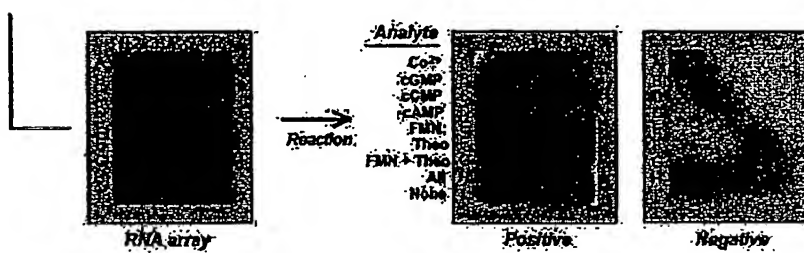
C.   **D.**   **E.**

FIGURE 66

A.

SPReeta SPR sensor

C.
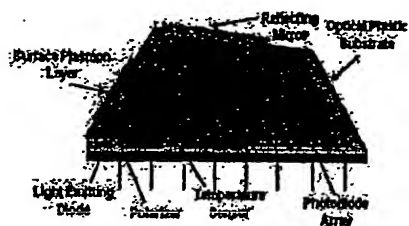
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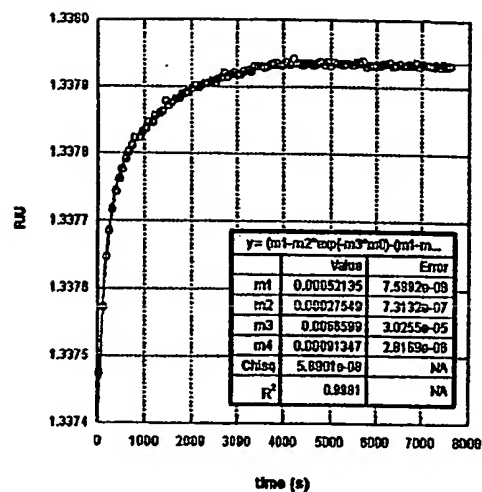
B.

HH NASM
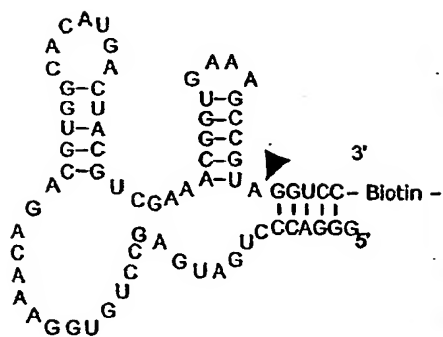
SEQ ID NO: 103 (same as cAMP)

Analyte-induced cleavage
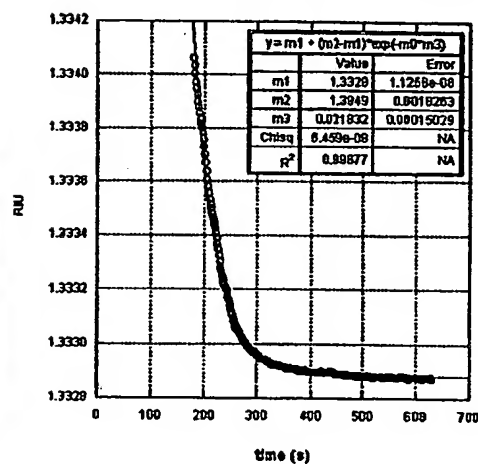
D.

FIGURE 67

cGMP (90 nt)   SEQ ID NO: 135

5' thiol-GGAUAAUAGCCGUAGGUUGCGAAAGCGACCCUGAUGA
GCCCUGCGAUGCAGAAAGGUGCUGACGACACAUCGAAACGGUAGCGAGAG
CUC 3'

cCMP (88 nt):   SEQ ID NO: 136

5' thiol-GGAUAAUAGCCGUAGGUUGCGAAAGCGACCCUGAUGA
CCUGUGGAAACAGACGUGGCACAUGACUACGU
GAAACGGUAGCGAGAGCUC 3'

cAMP (88 nt):   SEQ ID NO: 137

5' thiol- GGAUAAUAGCCGUAGGUUGCGAAAGCGACCCUGAUGA
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SEQ ID NO: 137
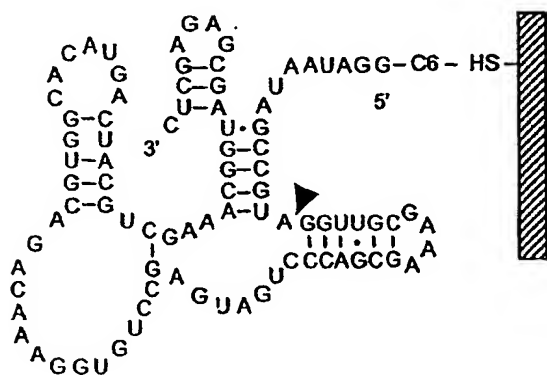
FIGURE 68

cCMP ((71 nt)):   SEQ ID NO: 138
5' GGGACCCUGAUGAG CCUUUAGGGCCAAGUGUGGUGAAAGACACACGU
CGAAACGGUGAAAGCCGUAGGUCC-Biotin 3'

cAMP ((70 nt):   SEQ ID NO: 103
5' GGGACCCUGAUGAG CCUGUGGAAACAGACGUGGCACAUGACUACGU·
CGAAACGGUGAAAGCCGUAGGUCC-Biotin 3'

cGMP (70 nt):   SEQ ID NO: 139
5' GGGACCCUGAUGAG CCUUGCGAUGCAAAAAGGUGCUGACGACACAU
CGAAACGGUGAAAGCCGUAGGUCC-Biotin 3'

SEQ ID NO: 103
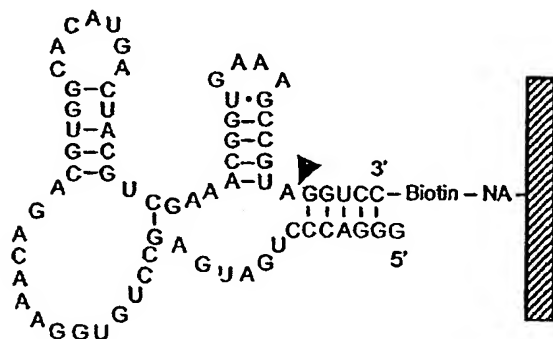
FIGURE 69

cCMP: SEQ ID NO: 40

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cAMP: SEQ ID NO: 41

5' GGACCCUGAUGAG CCUGUGGAAACAGACGUGGCACAUGACUACGU CGAAACGGUGAAAGCCGUAGGUCCUUGCGUGGUUCUGUUCCCUUCUUCG 3'

cGMP: SEQ ID NO: 42

5' GGACCCUGAUGAG CCUUGCGAUGCAAAAAGGUGCUGACGACACAU CGAAACGGUGAAAGCCGUAGGUCCUUGCGUGGUUCUGUUCCCUUCUUCG 3'

Capture Oligo: SEQ ID NO: 43

3'-ACGCACCAAGACAAGGGAAGAAGC-Biotin-5'
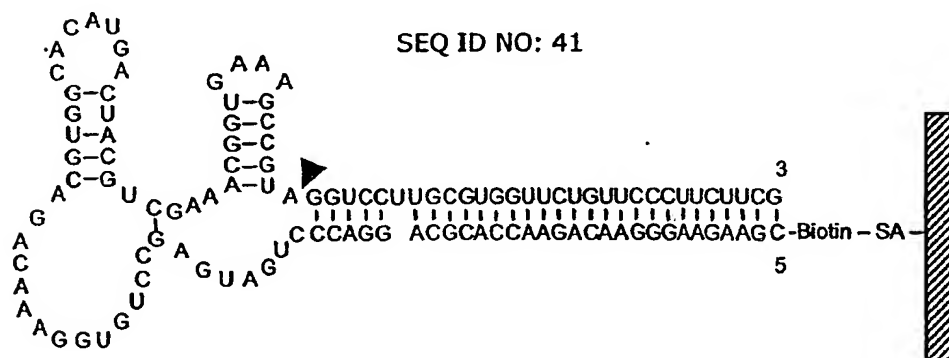
FIGURE 70

A. cGMP HH Binding to neutravidin-gold surface
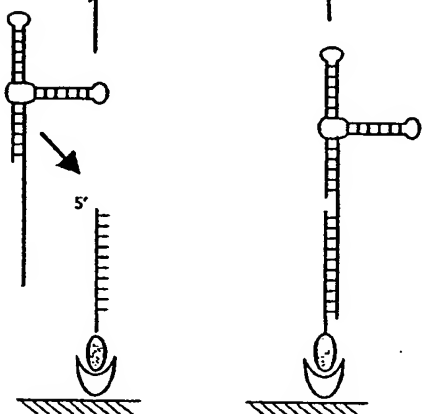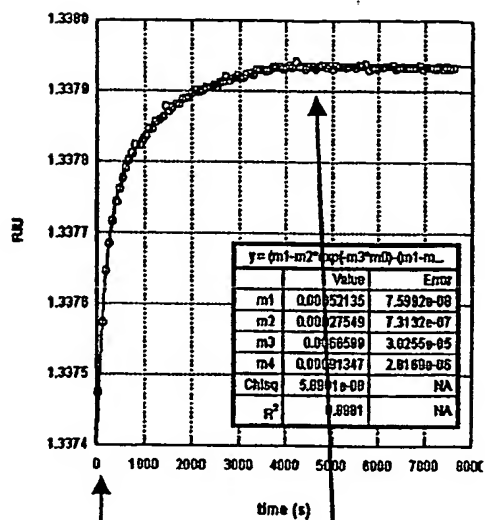
B. cGMP HH cleavage with 1 mM cGMP

C.

$K_{ads} \sim 0.41\ min^{-1}$
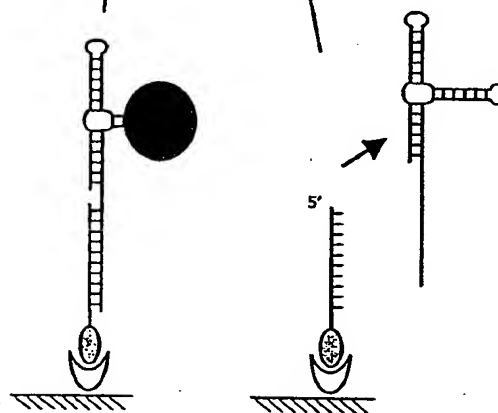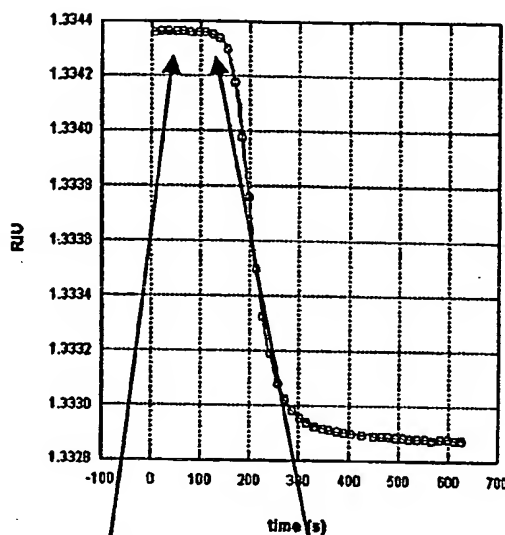
$K_{clv} \sim 1.31\ min^{-1}$

FIGURE 71
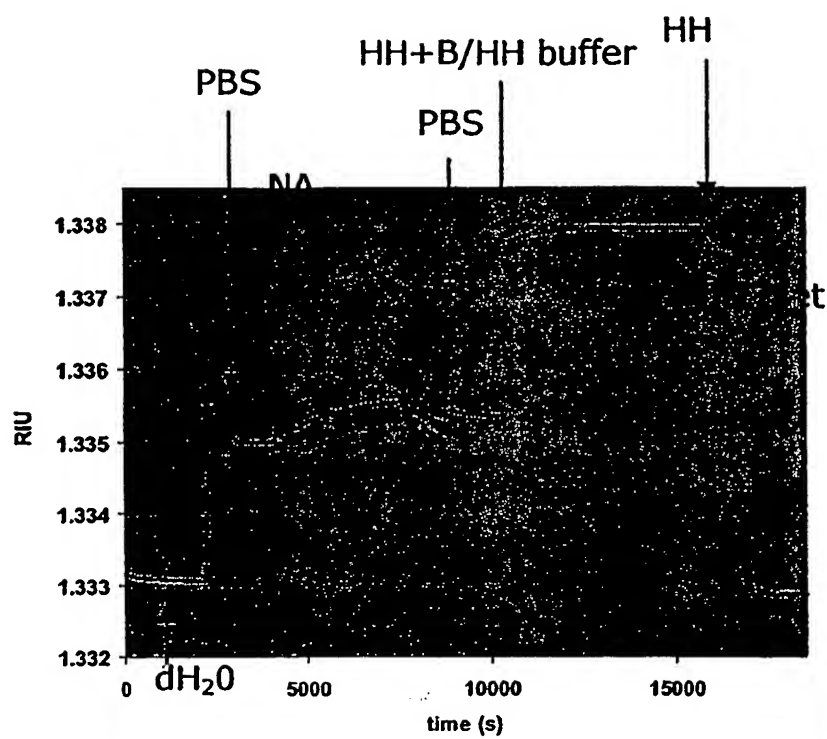
FIGURE 72
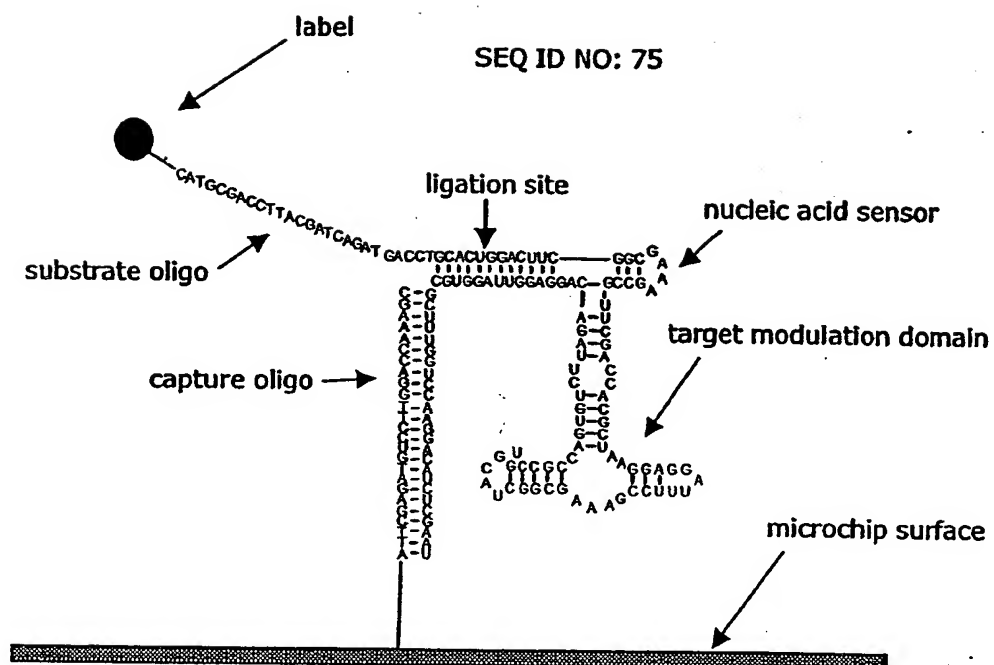
FIGURE 73
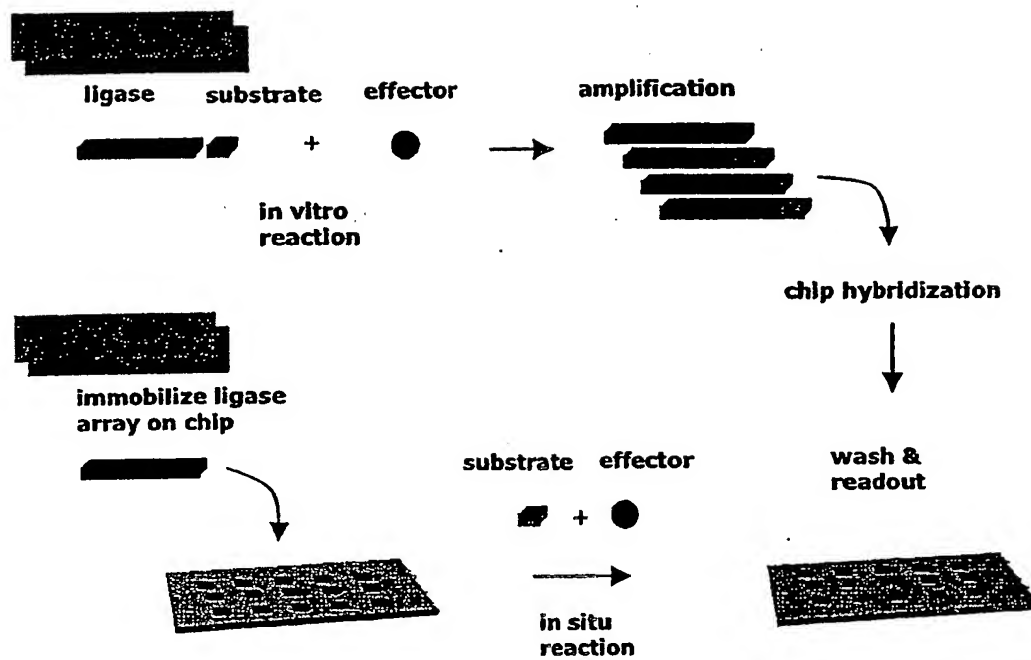
FIGURE 74

Figure 75

A   Hybridized & Washed
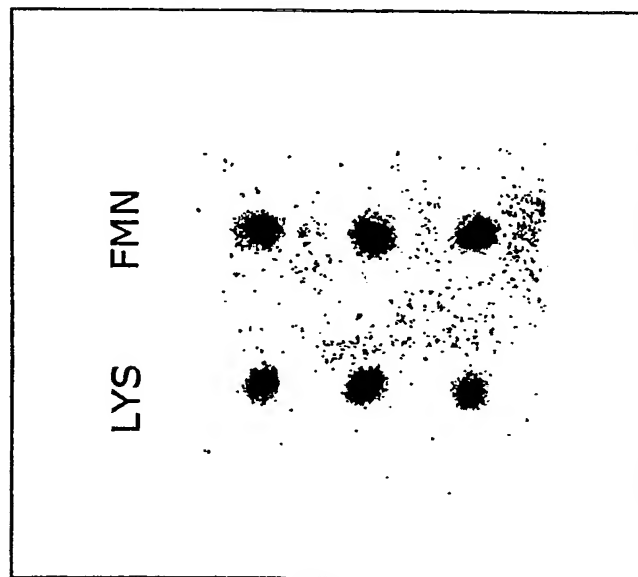
B   Ligated & Denatured

FIGURE 76

A

[ERK], µM →

B
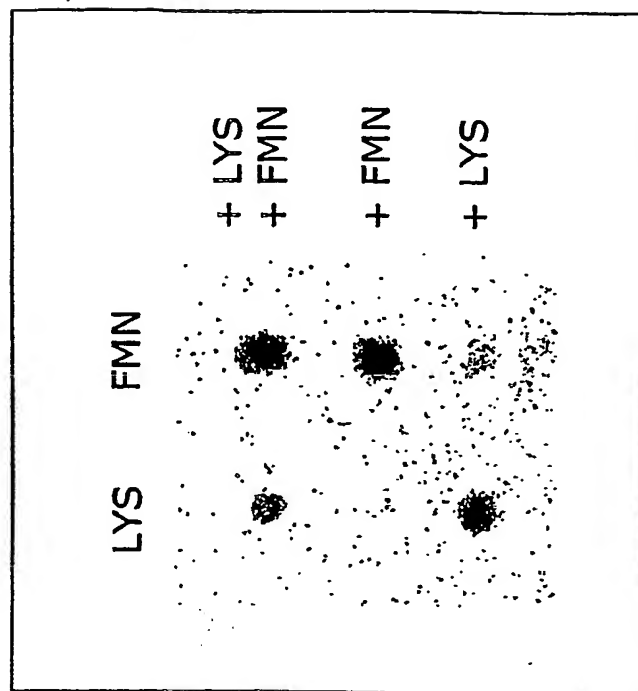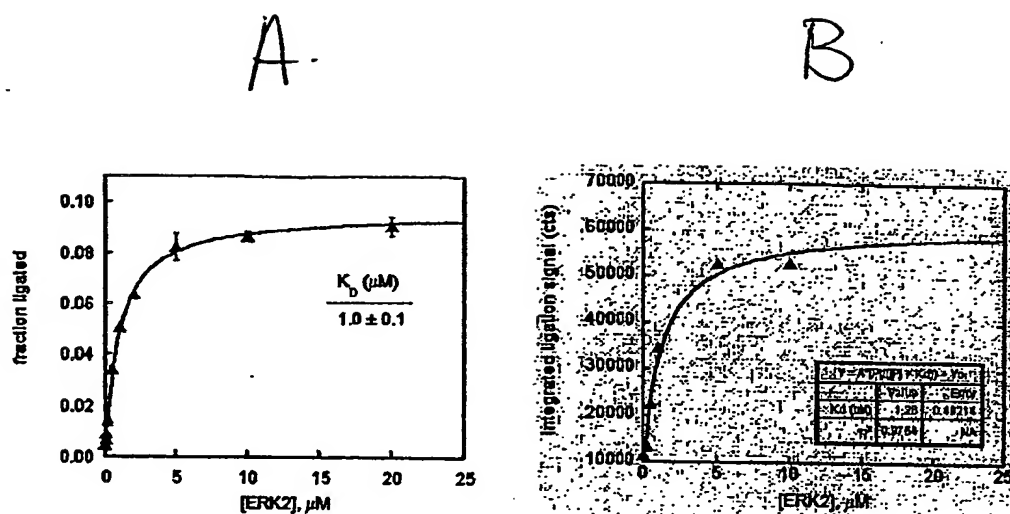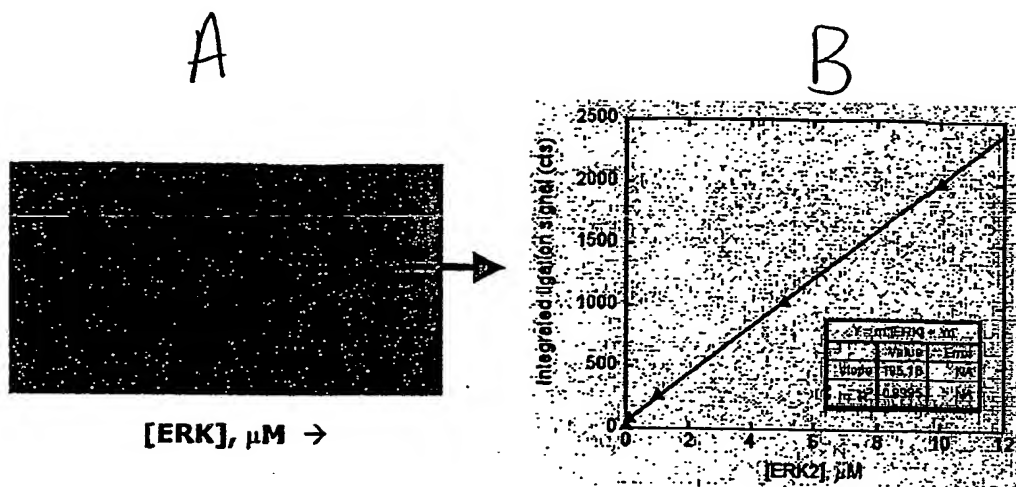
FIGURE 77

A.

**Captured Cy3-ligase:
100% signal**

**TSA-Cy5 substrate:
ligation signal**

B.

FIGURE 78

FIGURE 79

set of 10 arbitrary
sequence tags
specific to one
analyte sensor

10 unique
analyte
sensors

5'   3'

analyte   capture   RT primer
sensing   binding
domain   site

FIGURE 80

T7 promoter
RT primer

● Analyte activates ligase

↓ RT extension

↓ Base treatment to hydrolyze RNA

↓ Transcription with labeled NTPs

↓ Hybridization to capture chip

In the absence of analyte, no ligation takes place, T7 promoter remains single stranded and unlinked to template → no transcription, no labeling

DNA
RNA

FIGURE 81

Figure 82

SEQUENCE LISTING

<110> Archemix Corporation
     Stanton, Martin
     Epstein, David
     Hamaguchi, Nobuko
     Kurz, Markus
     Keefe, Tony
     Wilson, Charles
     Grate, Dilara
     Marshall, Kristin
     McCauley, Thomas
     Kurz, Jeffrey

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<140> Not yet assigned
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            20                  25                  30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
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Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
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Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
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Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
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Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
        115             120             125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
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Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145             150             155             160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
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Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
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Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
        195             200             205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
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Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
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Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
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Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
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Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
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Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
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Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
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5

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Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
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Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
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Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
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Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
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<212> PRT
<213> Homo sapiens

<400> 12
Met Gly Gln Pro Gly Asn Gly Ser Ala Phe Leu Leu Ala Pro Asn Gly
  1               5                  10                  15

Ser His Ala Pro Asp His Asp Val Thr Gln Gln Arg Asp Glu Val Trp
             20                  25                  30

Val Val Gly Met Gly Ile Val Met Ser Leu Ile Val Leu Ala Ile Val
         35                  40                  45

Phe Gly Asn Val Leu Val Ile Thr Ala Ile Ala Lys Phe Glu Arg Leu
     50                  55                  60

Gln Thr Val Thr Asn Tyr Phe Ile Thr Ser Leu Ala Cys Ala Asp Leu
 65                  70                  75                  80

Val Met Gly Leu Ala Val Val Pro Phe Gly Ala Ala His Ile Leu Met
                 85                  90                  95

Lys Met Trp Thr Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr Ser Ile
                100                 105                 110

Asp Val Leu Cys Val Thr Ala Ser Ile Glu Thr Leu Cys Val Ile Ala
             115                 120                 125

Val Asp Arg Tyr Phe Ala Ile Thr Ser Pro Phe Lys Tyr Gln Ser Leu
         130                 135                 140

Leu Thr Lys Asn Lys Ala Arg Val Ile Ile Leu Met Val Trp Ile Val
145                 150                 155                 160

Ser Gly Leu Thr Ser Phe Leu Pro Ile Gln Met His Trp Tyr Arg Ala
```

```
                165                    170                    175
Thr His Gln Glu Ala Ile Asn Cys Tyr Ala Asn Glu Thr Cys Cys Asp
            180                    185                    190
Phe Phe Thr Asn Gln Ala Tyr Ala Ile Ala Ser Ser Ile Val Ser Phe
            195                    200                    205
Tyr Val Pro Leu Val Ile Met Val Phe Val Tyr Ser Arg Val Phe Gln
210                    215                    220
Glu Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys Ser Glu Gly Arg Phe
225                    230                    235                    240
His Val Gln Asn Leu Ser Gln Val Glu Gln Asp Gly Arg Thr Gly His
            245                    250                    255
Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala Leu
            260                    265                    270
Lys Thr Leu Gly Ile Ile Met Gly Thr Phe Thr Leu Cys Trp Leu Pro
            275                    280                    285
Phe Phe Ile Val Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Arg
            290                    295                    300
Lys Glu Val Tyr Ile Leu Leu Asn Trp Ile Gly Tyr Val Asn Ser Gly
305                    310                    315                    320
Phe Asn Pro Leu Ile Tyr Cys Arg Ser Pro Asp Phe Arg Ile Ala Phe
            325                    330                    335
Gln Glu Leu Leu Cys Leu Arg Arg Ser Ser Leu Lys Ala Tyr Gly Asn
            340                    345                    350
Gly Tyr Ser Ser Asn Gly Asn Thr Gly Glu Gln Ser Gly Tyr His Val
            355                    360                    365
Glu Gln Glu Lys Glu Asn Lys Leu Leu Cys Glu Asp Leu Pro Gly Thr
            370                    375                    380
Glu Asp Phe Val Gly His Gln Gly Thr Val Pro Ser Asp Asn Ile Asp
385                    390                    395                    400
Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp Ser Leu Leu
            405                    410
```

```
<210> 13
<211> 394
<212> PRT
<213> Homo sapiens

<400> 13
Met Gly Cys Leu Gly Asn Ser Lys Thr Glu Asp Gln Arg Asn Glu Glu
1               5                   10                  15

Lys Ala Gln Arg Glu Ala Asn Lys Lys Ile Glu Lys Gln Leu Gln Lys
            20                  25                  30

Asp Lys Gln Val Tyr Arg Ala Thr His Arg Leu Leu Leu Leu Gly Ala
```

```
              35                    40                    45
Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Arg Ile Leu His
    50                  55                  60

Val Asn Gly Phe Asn Gly Glu Gly Gly Glu Glu Asp Pro Gln Ala Ala
65                  70                  75                  80

Arg Ser Asn Ser Asp Gly Glu Lys Ala Thr Lys Val Gln Asp Ile Lys
                85                  90                  95

Asn Asn Leu Lys Glu Ala Ile Glu Thr Ile Val Ala Ala Met Ser Asn
            100                 105                 110

Leu Val Pro Pro Val Glu Leu Ala Asn Pro Glu Asn Gln Phe Arg Val
        115                 120                 125

Asp Tyr Ile Leu Ser Val Met Asn Val Pro Asp Phe Asp Phe Pro Pro
    130                 135                 140

Glu Phe Tyr Glu His Ala Lys Ala Leu Trp Glu Asp Glu Gly Val Arg
145                 150                 155                 160

Ala Cys Tyr Glu Arg Ser Asn Glu Tyr Gln Leu Ile Asp Cys Ala Gln
            165                 170                 175

Tyr Phe Leu Asp Lys Ile Asp Val Ile Lys Gln Ala Asp Tyr Val Pro
            180                 185                 190

Ser Asp Gln Asp Leu Leu Arg Cys Arg Val Leu Thr Ser Gly Ile Phe
    195                 200                 205

Glu Thr Lys Phe Gln Val Asp Lys Val Asn Phe His Met Phe Asp Val
    210                 215                 220

Gly Gly Gln Arg Asp Glu Arg Arg Lys Trp Ile Gln Cys Phe Asn Asp
225                 230                 235                 240

Val Thr Ala Ile Ile Phe Val Val Ala Ser Ser Ser Tyr Asn Met Val
            245                 250                 255

Ile Arg Glu Asp Asn Gln Thr Asn Arg Leu Gln Glu Ala Leu Asn Leu
            260                 265                 270

Phe Lys Ser Ile Trp Asn Asn Arg Trp Leu Arg Thr Ile Ser Val Ile
        275                 280                 285

Leu Phe Leu Asn Lys Gln Asp Leu Leu Ala Glu Lys Val Leu Ala Gly
        290                 295                 300

Lys Ser Lys Ile Glu Asp Tyr Phe Pro Glu Phe Ala Arg Tyr Thr Thr
305                 310                 315                 320

Pro Glu Asp Ala Thr Pro Glu Pro Gly Glu Asp Pro Arg Val Thr Arg
                325                 330                 335

Ala Lys Tyr Phe Ile Arg Asp Glu Phe Leu Arg Ile Ser Thr Ala Ser
            340                 345                 350

Gly Asp Gly Arg His Tyr Cys Tyr Pro His Phe Thr Cys Ala Val Asp
    355                 360                 365
```

9

```
Thr Glu Asn Ile Arg Arg Val Phe Asn Asp Cys Arg Asp Ile Ile Gln
    370                 375             380

Arg Met His Leu Arg Gln Tyr Glu Leu Leu
385                 390


<210> 14
<211> 395
<212> PRT
<213> Homo sapiens

<400> 14
Met Gly Cys Leu Gly Asn Ser Lys Thr Glu Asp Gln Arg Asn Glu Glu
  1             5             10                15

Lys Ala Gln Arg Glu Ala Asn Lys Lys Ile Glu Lys Gln Leu Gln Lys
                20              25              30

Asp Lys Gln Val Tyr Arg Ala Thr His Arg Leu Leu Leu Leu Gly Ala
            35              40              45

Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Arg Ile Leu His
        50              55              60

Val Asn Gly Phe Asn Gly Glu Gly Gly Glu Glu Asp Pro Gln Ala Ala
 65             70              75                  80

Arg Ser Asn Ser Asp Gly Ser Glu Lys Ala Thr Lys Val Gln Asp Ile
                85              90              95

Lys Asn Asn Leu Lys Glu Ala Ile Glu Thr Ile Val Ala Ala Met Ser
            100             105             110

Asn Leu Val Pro Pro Val Glu Leu Ala Asn Pro Glu Asn Gln Phe Arg
        115             120             125

Val Asp Tyr Ile Leu Ser Val Met Asn Val Pro Asp Phe Asp Phe Pro
    130             135              140

Pro Glu Phe Tyr Glu His Ala Lys Ala Leu Trp Glu Asp Glu Gly Val
145             150             155             160

Arg Ala Cys Tyr Glu Arg Ser Asn Glu Tyr Gln Leu Ile Asp Cys Ala
            165             170             175

Gln Tyr Phe Leu Asp Lys Ile Asp Val Ile Lys Gln Ala Asp Tyr Val
        180             185             190

Pro Ser Asp Gln Asp Leu Leu Arg Cys Arg Val Leu Thr Ser Gly Ile
        195             200             205

Phe Glu Thr Lys Phe Gln Val Asp Lys Val Asn Phe His Met Phe Asp
    210             215             220

Val Gly Gly Gln Arg Asp Glu Arg Arg Lys Trp Ile Gln Cys Phe Asn
225             230             235             240

Asp Val Thr Ala Ile Ile Phe Val Val Ala Ser Ser Ser Tyr Asn Met
            245             250             255
```

                                   10

```
Val Ile Arg Glu Asp Asn Gln Thr Asn Arg Leu Gln Glu Ala Leu Asn
            260                 265             270

Leu Phe Lys Ser Ile Trp Asn Asn Arg Trp Leu Arg Thr Ile Ser Val
            275                 280             285

Ile Leu Phe Leu Asn Lys Gln Asp Leu Leu Ala Glu Lys Val Leu Ala
            290                 295             300

Gly Lys Ser Lys Ile Glu Asp Tyr Phe Pro Glu Phe Ala Arg Tyr Thr
305             310             315             320

Thr Pro Glu Asp Ala Thr Pro Glu Pro Gly Glu Asp Pro Arg Val Thr
            325             330             335

Arg Ala Lys Tyr Phe Ile Arg Asp Glu Phe Leu Arg Ile Ser Thr Ala
            340             345             350

Ser Gly Asp Gly Arg His Tyr Cys Tyr Pro His Phe Thr Cys Ala Val
            355             360             365

Asp Thr Glu Asn Ile Arg Arg Val Phe Asn Asp Cys Arg Asp Ile Ile
    370             375             380

Gln Arg Met His Leu Arg Gln Tyr Glu Leu Leu
385             390             395


<210> 15
<211> 379
<212> PRT
<213> Homo sapiens

<400> 15
Met Gly Cys Leu Gly Asn Ser Lys Thr Glu Asp Gln Arg Asn Glu Glu
  1             5               10              15

Lys Ala Gln Arg Glu Ala Asn Lys Lys Ile Glu Lys Gln Leu Gln Lys
            20              25              30

Asp Lys Gln Val Tyr Arg Ala Thr His Arg Leu Leu Leu Leu Gly Ala
        35              40              45

Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Arg Ile Leu His
    50              55              60

Val Asn Gly Phe Asn Gly Asp Glu Lys Ala Thr Lys Val Gln Asp Ile
65              70              75              80

Lys Asn Asn Leu Lys Glu Ala Ile Glu Thr Ile Val Ala Ala Met Ser
            85              90              95

Asn Leu Val Pro Pro Val Glu Leu Ala Asn Pro Glu Asn Gln Phe Arg
            100             105             110

Val Asp Tyr Ile Leu Ser Val Met Asn Val Pro Asp Phe Asp Phe Pro
        115             120             125

Pro Glu Phe Tyr Glu His Ala Lys Ala Leu Trp Glu Asp Glu Gly Val
    130             135             140
```

```
Arg Ala Cys Tyr Glu Arg Ser Asn Glu Tyr Gln Leu Ile Asp Cys Ala
145             150             155             160

Gln Tyr Phe Leu Asp Lys Ile Asp Val Ile Lys Gln Ala Asp Tyr Val
                165             170             175

Pro Ser Asp Gln Asp Leu Leu Arg Cys Arg Val Leu Thr Ser Gly Ile
            180             185             190

Phe Glu Thr Lys Phe Gln Val Asp Lys Val Asn Phe His Met Phe Asp
        195             200             205

Val Gly Gly Gln Arg Asp Glu Arg Arg Lys Trp Ile Gln Cys Phe Asn
    210             215             220

Asp Val Thr Ala Ile Ile Phe Val Val Ala Ser Ser Ser Tyr Asn Met
225             230             235             240

Val Ile Arg Glu Asp Asn Gln Thr Asn Arg Leu Gln Glu Ala Leu Asn
            245             250             255

Leu Phe Lys Ser Ile Trp Asn Asn Arg Trp Leu Arg Thr Ile Ser Val
            260             265             270

Ile Leu Phe Leu Asn Lys Gln Asp Leu Leu Ala Glu Lys Val Leu Ala
            275             280             285

Gly Lys Ser Lys Ile Glu Asp Tyr Phe Pro Glu Phe Ala Arg Tyr Thr
    290             295             300

Thr Pro Glu Asp Ala Thr Pro Glu Pro Gly Glu Asp Pro Arg Val Thr
305             310             315             320

Arg Ala Lys Tyr Phe Ile Arg Asp Glu Phe Leu Arg Ile Ser Thr Ala
            325             330             335

Ser Gly Asp Gly Arg His Tyr Cys Tyr Pro His Phe Thr Cys Ala Val
            340             345             350

Asp Thr Glu Asn Ile Arg Arg Val Phe Asn Asp Cys Arg Asp Ile Ile
        355             360             365

Gln Arg Met His Leu Arg Gln Tyr Glu Leu Leu
    370             375


<210> 16
<211> 380
<212> PRT
<213> Homo sapiens

<400> 16
Met Gly Cys Leu Gly Asn Ser Lys Thr Glu Asp Gln Arg Asn Glu Glu
1               5               10              15

Lys Ala Gln Arg Glu Ala Asn Lys Lys Ile Glu Lys Gln Leu Gln Lys
            20              25              30

Asp Lys Gln Val Tyr Arg Ala Thr His Arg Leu Leu Leu Leu Gly Ala
        35              40              45
```

```
Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Arg Ile Leu His
    50              55              60

Val Asn Gly Phe Asn Gly Asp Ser Glu Lys Ala Thr Lys Val Gln Asp
65              70              75              80

Ile Lys Asn Asn Leu Lys Glu Ala Ile Glu Thr Ile Val Ala Ala Met
            85              90              95

Ser Asn Leu Val Pro Pro Val Glu Leu Ala Asn Pro Glu Asn Gln Phe
            100             105             110

Arg Val Asp Tyr Ile Leu Ser Val Met Asn Val Pro Asp Phe Asp Phe
        115             120             125

Pro Pro Glu Phe Tyr Glu His Ala Lys Ala Leu Trp Glu Asp Glu Gly
    130             135             140

Val Arg Ala Cys Tyr Glu Arg Ser Asn Glu Tyr Gln Leu Ile Asp Cys
145             150             155             160

Ala Gln Tyr Phe Leu Asp Lys Ile Asp Val Ile Lys Gln Ala Asp Tyr
            165             170             175

Val Pro Ser Asp Gln Asp Leu Leu Arg Cys Arg Val Leu Thr Ser Gly
        180             185             190

Ile Phe Glu Thr Lys Phe Gln Val Asp Lys Val Asn Phe His Met Phe
        195             200             205

Asp Val Gly Gly Gln Arg Asp Glu Arg Arg Lys Trp Ile Gln Cys Phe
    210             215             220

Asn Asp Val Thr Ala Ile Ile Phe Val Val Ala Ser Ser Ser Tyr Asn
225             230             235             240

Met Val Ile Arg Glu Asp Asn Gln Thr Asn Arg Leu Gln Glu Ala Leu
            245             250             255

Asn Leu Phe Lys Ser Ile Trp Asn Asn Arg Trp Leu Arg Thr Ile Ser
        260             265             270

Val Ile Leu Phe Leu Asn Lys Gln Asp Leu Leu Ala Glu Lys Val Leu
        275             280             285

Ala Gly Lys Ser Lys Ile Glu Asp Tyr Phe Pro Glu Phe Ala Arg Tyr
    290             295             300

Thr Thr Pro Glu Asp Ala Thr Pro Glu Pro Gly Glu Asp Pro Arg Val
305             310             315             320

Thr Arg Ala Lys Tyr Phe Ile Arg Asp Glu Phe Leu Arg Ile Ser Thr
            325             330             335

Ala Ser Gly Asp Gly Arg His Tyr Cys Tyr Pro His Phe Thr Cys Ala
            340             345             350

Val Asp Thr Glu Asn Ile Arg Arg Val Phe Asn Asp Cys Arg Asp Ile
    355             360             365
```

13

Ile Gln Arg Met His Leu Arg Gln Tyr Glu Leu Leu
    370   375   380

<210> 17
<211>' 388
<212> PRT
<213> Homo sapiens

<400> 17
Met Glu Ile Ser Gly Pro Pro Phe Glu Ile Gly Ser Ala Pro Ala Gly
  1   5   10   15

Val Asp Asp Thr Pro Val Asn Met Asp Ser Pro Pro Ile Ala Leu Asp
             20   25   30

Gly Pro Pro Ile Lys Val Ser Gly Ala Pro Asp Lys Arg Glu Arg Ala
         35   40   45

Glu Arg Pro Pro Val Glu Glu Glu Ala Ala Glu Met Glu Gly Ala Ala
     50   '   55   60

Asp Ala Ala Glu Gly Gly Lys Val Pro Ser Pro Gly Tyr Gly Ser Pro
 65   70   75   80

Ala Ala Gly Ala Ala Ser Ala Asp Thr Ala Ala Arg Ala Ala Pro Ala
             85   90   95

Ala Pro Ala Asp Pro Asp Ser Gly Ala Thr Pro Glu Asp Pro Asp Ser
         100   105   110

Gly Thr Ala Pro Ala Asp Pro Asp Ser Gly Ala Phe Ala Ala Asp Pro
     115   120   125

Asp Ser Gly Ala Ala Pro Ala Ala Pro Ala Asp Pro Asp Ser Gly Ala
     130   135   140

Ala Pro Asp Ala Pro Ala Asp Pro Asp Ser Gly Ala Ala Pro Asp Ala
145   150   155   160

Pro Ala Asp Pro Asp Ala Gly Ala Ala Pro Glu Ala Pro Ala Ala Pro
         165   170   175

Ala Ala Ala Glu Thr Arg Ala Ala His Val Ala Pro Ala Ala Pro Asp
             180   185   190

Ala Gly Ala Pro Thr Ala Pro Ala Ala Ser Ala Thr Arg Ala Ala Gln
         195   200   205

Val Arg Arg Ala Ala Ser Ala Ala Pro Ala Ser Gly Ala Arg Arg Lys
     210   215   220

Ile His Leu Arg Pro Pro Ser Pro Glu Ile Gln Ala Ala Asp Pro Pro
225   230   235   240

Thr Pro Arg Pro Thr Arg Ala Ser Ala Trp Arg Gly Lys Ser Glu Ser
             245   250   255

Ser Arg Gly Arg Arg Val Tyr Tyr Asp Glu Gly Val Ala Ser Ser Asp
         260   265   270

14

```
Asp Asp Ser Ser Gly Asp Glu Ser Asp Asp Gly Thr Ser Gly Cys Leu
        275             280             285

Arg Trp Phe Gln His Arg Arg Asn Arg Arg Arg Arg Lys Pro Gln Arg
    290             295             300

Asn Leu Leu Arg Asn Phe Leu Val Gln Ala Phe Gly Gly Cys Phe Gly
305             310             315             320

Arg Ser Glu Ser Pro Gln Pro Lys Ala Ser Arg Ser Leu Lys Val Lys
            325             330             335

Lys Val Pro Leu Ala Glu Lys Arg Arg Gln Met Arg Lys Glu Ala Leu
        340             345             350

Glu Lys Arg Ala Gln Lys Arg Ala Glu Lys Lys Arg Ser Lys Leu Ile
    355             360             365

Asp Lys Gln Leu Gln Asp Glu Lys Met Gly Tyr Met Cys Thr His Arg
    370             375             380

Leu Leu Leu Leu
385


<210> 18
<211> 381
<212> PRT
<213> Homo sapiens

<400> 18
Met Gly Cys Leu Gly Gly Asn Ser Lys Thr Thr Glu Asp Gln Gly Val
 1               5              10              15

Asp Glu Lys Glu Arg Arg Glu Ala Asn Lys Lys Ile Glu Lys Gln Leu
        20              25              30

Gln Lys Glu Arg Leu Ala Tyr Lys Ala Thr His Arg Leu Leu Leu Leu
        35              40              45

Gly Ala Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Arg Ile
    50              55              60

Leu His Val Asn Gly Phe Asn Pro Glu Glu Lys Lys Gln Lys Ile Leu
65              70              75              80

Asp Ile Arg Lys Asn Val Lys Asp Ala Ile Val Thr Ile Val Ser Ala
            85              90              95

Met Ser Thr Ile Ile Pro Pro Val Pro Leu Ala Asn Pro Glu Asn Gln
            100             105             110

Phe Arg Ser Asp Tyr Ile Lys Ser Ile Ala Pro Ile Thr Asp Phe Glu
    115             120             125

Tyr Ser Gln Glu Phe Phe Asp His Val Lys Lys Leu Trp Asp Asp Glu
    130             135             140

Gly Val Lys Ala Cys Phe Glu Arg Ser Asn Glu Tyr Gln Leu Ile Asp
145             150             155             160
```

```
Cys Ala Gln Tyr Phe Leu Glu Arg Ile Asp Ser Val Ser Leu Val Asp
              165             170             175

Tyr Thr Pro Thr Asp Gln Asp Leu Leu Arg Cys Arg Val Leu Thr Ser
          180             185             190

Gly Ile Phe Glu Thr Arg Phe Gln Val Asp Lys Val Asn Phe His Met
          195             200             205

Phe Asp Val Gly Gly Gln Arg Asp Glu Arg Arg Lys Trp Ile Gln Cys
      210             215             220

Phe Asn Asp Val Thr Ala Ile Ile Tyr Val Ala Ala Cys Ser Ser Tyr
225             230             235             240

Asn Met Val Ile Arg Glu Asp Asn Asn Thr Asn Arg Leu Arg Glu Ser
              245             250             255

Leu Asp Leu Phe Glu Ser Ile Trp Asn Asn Arg Trp Leu Arg Thr Ile
          260             265             270

Ser Ile Ile Leu Phe Leu Asn Lys Gln Asp Met Leu Ala Glu Lys Val
          275             280             285

Leu Ala Gly Lys Ser Lys Ile Glu Asp Tyr Phe Pro Glu Tyr Ala Asn
      290             295             300

Tyr Thr Val Pro Glu Asp Ala Thr Pro Asp Ala Gly Glu Asp Pro Lys
305             310             315             320

Val Thr Arg Ala Lys Phe Phe Ile Arg Asp Leu Phe Leu Arg Ile Ser
              325             330             335

Thr Ala Thr Gly Asp Gly Lys His Tyr Cys Tyr Pro His Phe Thr Cys
              340             345             350

Ala Val Asp Thr Glu Asn Ile Arg Arg Val Phe Asn Asp Cys Arg Asp
      355             360             365

Ile Ile Gln Arg Met His Leu Lys Gln Tyr Glu Leu Leu
    370             375             380
```

```
<210> 19
<211> 354
<212> PRT
<213> Homo sapiens

<400> 19
Met Gly Cys Thr Leu Ser Ala Glu Asp Lys Ala Ala Val Glu Arg Ser
  1           5               10              15

Lys Met Ile Asp Arg Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Arg
              20              25              30

Glu Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
          35              40              45

Ile Val Lys Gln Met Lys Ile Ile His Glu Ala Gly Tyr Ser Glu Glu
      50              55              60
```

Glu Cys Lys Gln Tyr Lys Ala Val Val Tyr Ser Asn Thr Ile Gln Ser
65   70   75   80

Ile Ile Ala Ile Ile Arg Ala Met Gly Arg Leu Lys Ile Asp Phe Gly
                85   90   95

Asp Ser Ala Arg Ala Asp Asp Ala Arg Gln Leu Phe Val Leu Ala Gly
            100   105   110

Ala Ala Glu Glu Gly Phe Met Thr Ala Glu Leu Ala Gly Val Ile Lys
        115   120   125

Arg Leu Trp Lys Asp Ser Gly Val Gln Ala Cys Phe Asn Arg Ser Arg
    130   135   140

Glu Tyr Gln Leu Asn Asp Ser Ala Ala Tyr Tyr Leu Asn Asp Leu Asp
145   150   155   160

Arg Ile Ala Gln Pro Asn Tyr Ile Pro Thr Gln Gln Asp Val Leu Arg
            165   170   175

Thr Arg Val Lys Thr Thr Gly Ile Val Glu Thr His Phe Thr Phe Lys
        180   185   190

Asp Leu His Phe Lys Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg
        195   200   205

Lys Lys Trp Ile His Cys Phe Glu Gly Val Thr Ala Ile Ile Phe Cys
    210   215   220

Val Ala Leu Ser Asp Tyr Asp Leu Val Leu Ala Glu Asp Glu Glu Met
225   230   235   240

Asn Arg Met His Glu Ser Met Lys Leu Phe Asp Ser Ile Cys Asn Asn
            245   250   255

Lys Trp Phe Thr Asp Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys Asp
            260   265   270

Leu Phe Glu Glu Lys Ile Lys Lys Ser Pro Leu Thr Ile Cys Tyr Pro
        275   280   285

Glu Tyr Ala Gly Ser Asn Thr Tyr Glu Glu Ala Ala Ala Tyr Ile Gln
    290   295   300

Cys Gln Phe Glu Asp Leu Asn Lys Arg Lys Asp Thr Lys Glu Ile Tyr
305   310   315   320

Thr His Phe Thr Cys Ala Thr Asp Thr Lys Asn Val Gln Phe Val Phe
            325   330   335

Asp Ala Val Thr Asp Val Ile Ile Lys Asn Asn Leu Lys Asp Cys Gly
            340   345   350

Leu Phe

<210> 20
<211> 355
<212> PRT

17

<213> Homo sapiens

<400> 20

```
Met Gly Cys Thr Val Ser Ala Glu Asp Lys Ala Ala Ala Glu Arg Ser
 1               5                  10                 15

Lys Met Ile Asp Lys Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Arg
           20                  25                 30

Glu Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
           35                  40                 45

Ile Val Lys Gln Met Lys Ile Ile His Glu Asp Gly Tyr Ser Glu Glu
       50                  55                 60

Glu Cys Arg Gln Tyr Arg Ala Val Val Tyr Ser Asn Thr Ile Gln Ser
65                  70                 75                 80

Ile Met Ala Ile Val Lys Ala Met Gly Asn Leu Gln Ile Asp Phe Ala
               85                  90                 95

Asp Pro Ser Arg Ala Asp Asp Ala Arg Gln Leu Phe Ala Leu Ser Cys
           100                 105                110

Thr Ala Glu Glu Gln Gly Val Leu Pro Asp Asp Leu Ser Gly Val Ile
       115                 120                125

Arg Arg Leu Trp Ala Asp His Gly Val Gln Ala Cys Phe Gly Arg Ser
   130                 135                140

Arg Glu Tyr Gln Leu Asn Asp Ser Ala Ala Tyr Tyr Leu Asn Asp Leu
145                 150                155                160

Glu Arg Ile Ala Gln Ser Asp Tyr Ile Pro Thr Gln Gln Asp Val Leu
               165                 170                175

Arg Thr Arg Val Lys Thr Thr Gly Ile Val Glu Thr His Phe Thr Phe
           180                 185                190

Lys Asp Leu His Phe Lys Met Phe Asp Val Gly Gly Gln Arg Ser Glu
           195                 200                205

Arg Lys Lys Trp Ile His Cys Phe Glu Gly Val Thr Ala Ile Ile Phe
   210                 215                220

Cys Val Ala Leu Ser Ala Tyr Asp Leu Val Leu Ala Glu Asp Glu Glu
225                 230                235                240

Met Asn Arg Met His Glu Ser Met Lys Leu Phe Asp Ser Ile Cys Asn
               245                 250                255

Asn Lys Trp Phe Thr Asp Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys
           260                 265                270

Asp Leu Phe Glu Glu Lys Ile Thr His Ser Pro Leu Thr Ile Cys Phe
           275                 280                285

Pro Glu Tyr Thr Gly Ala Asn Lys Tyr Asp Glu Ala Ala Ser Tyr Ile
   290                 295                300

Gln Ser Lys Phe Glu Asp Leu Asn Lys Arg Lys Asp Thr Lys Glu Ile
```

```
        305                    310                    315                    320'
        Tyr Thr His Phe Thr Cys Ala Thr Asp Thr Lys Asn Val Gln Phe Val
                        325                    330                    335

        Phe Asp Ala Val Thr Asp Val Ile Ile Lys Asn Asn Leu Lys Asp Cys
                        340                    345                    350

        Gly Leu Phe
                355
```

<210> 21
<211> 354
<212> PRT
<213> Homo sapiens

<400> 21

```
        Met Gly Cys Thr Leu Ser Ala Glu Asp Lys Ala Ala Val Glu Arg Ser
          1               5                    10                    15

        Lys Met Ile Asp Arg Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Lys
                        20                .  25                    30

        Glu Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
                        35                    40                    45

        Ile Val Lys Gln Met Lys Ile Ile His Glu Asp Gly Tyr Ser Glu Asp
                        50                    55                    60

        Glu Cys Lys Gln Tyr Lys Val Val Val Tyr Ser Asn Thr Ile Gln Ser
         65                .  70                    75                    80

        Ile Ile Ala Ile Ile Arg Ala Met Gly Arg Leu Lys Ile Asp Phe Gly
              '                 85                    90                    95

        Glu Ala Ala Arg Ala Asp Asp Ala Arg Gln Leu Phe Val Leu Ala Gly
                        100                   105                   110

        Ser Ala Glu Glu Gly Val Met Thr Pro Glu Leu Ala Gly Val Ile Lys
                        115                   120                   125

        Arg Leu Trp Arg Asp Gly Gly Val Gln Ala Cys Phe Ser Arg Ser Arg
                130                   135                   140

        Glu Tyr Gln Leu Asn Asp Ser Ala Ser Tyr Tyr Leu Asn Asp Leu Asp
        ·145                   150                   155                   160

        Arg Ile Ser Gln Ser Asn Tyr Ile Pro Thr Gln Gln Asp Val Leu Arg
                        165                   170                   175

        Thr Arg Val Lys Thr Thr Gly Ile Val Glu Thr His Phe Thr Phe Lys
                        180                   185                   190

        Asp Leu Tyr Phe Lys Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg
                        195                   200                   205

        Lys Lys Trp Ile His Cys Phe Glu Gly Val Thr Ala Ile Ile Phe Cys
                210                   215                   220

        Val Ala Leu Ser Asp Tyr Asp Leu Val Leu Ala Glu Asp Glu Glu Met
```

19

```
      225                    230                    235                    240

      Asn Arg Met His Glu Ser Met Lys Leu Phe Asp Ser Ile Cys Asn Asn
                  245                250                    255

      Lys Trp Phe Thr Glu Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys Asp
                  260                265                270

      Leu Phe Glu Glu Lys Ile Lys Arg Ser Pro Leu Thr Ile Cys Tyr Pro
                  275                280                285

      Glu Tyr Thr Gly Ser Asn Thr Tyr Glu Glu Ala Ala Ala Tyr Ile Gln
                  290                295                300

      Cys Gln Phe Glu Asp Leu Asn Arg Arg Lys Asp Thr Lys Glu Ile Tyr
      305                310                315                    320

      Thr His Phe Thr Cys Ala Thr Asp Thr Lys Asn Val Gln Phe Val Phe
                      325                330                335

      Asp Ala Val Thr Asp Val Ile Ile Lys Asn Asn Leu Lys Glu Cys Gly
                  340                345                350

      Leu Tyr


      <210> 22
      <211> 354
      <212> PRT
      <213> Homo sapiens

      <400> 22
      Met Gly Cys Thr Leu Ser Ala Glu Glu Arg Ala Ala Leu Glu Arg Ser
      1               5                  10                     15

      Lys Ala Ile Glu Lys Asn Leu Lys Glu Asp Gly Ile Ser Ala Ala Lys
                  20                 25                 30

      Asp Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
                  35                 40                 45

      Ile Val Lys Gln Met Lys Ile Ile His Glu Asp Gly Phe Ser Gly Glu
                  50                 55                 60

      Asp Val Lys Gln Tyr Lys Pro Val Val Tyr Ser Asn Thr Ile Gln Ser
      65                 70                 75                     80

      Leu Ala Ala Ile Val Arg Ala Met Asp Thr Leu Gly Ile Glu Tyr Gly
                      85                 90                 95

      Asp Lys Glu Arg Lys Ala Asp Ala Lys Met Val Cys Asp Val Val Ser
                  100                105                110

      Arg Met Glu Asp Thr Glu Pro Phe Ser Ala Glu Leu Leu Ser Ala Met
                  115                120                125

      Met Arg Leu Trp Gly Asp Ser Gly Ile Gln Glu Cys Phe Asn Arg Ser
                  130                135                140

      Arg Glu Tyr Gln Leu Asn Asp Ser Ala Lys Tyr Tyr Leu Asp Ser Leu
```

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        145                 150                 155                 160

Asp Arg Ile Gly Ala Ala Asp Tyr Gln Pro Thr Glu Gln Asp Ile Leu
                165                 170                 175

Arg Thr Arg Val Lys Thr Thr Gly Ile Val Glu Thr His Phe Thr Phe
                180                 185                 190

Lys Asn Leu His Phe Arg Leu Phe Asp Val Gly Gly Gln Arg Ser Glu
                195                 200                 205

Arg Lys Lys Trp Ile His Cys Phe Glu Asp Val Thr Ala Ile Ile Phe
        210                 215                 220

Cys Val Ala Leu Ser Gly Tyr Asp Gln Val Leu His Glu Asp Glu Thr
225                 230                 235                 240

Thr Asn Arg Met His Glu Ser Leu Met Leu Phe Asp Ser Ile Cys Asn
                245                 250                 255

Asn Lys Phe Phe Ile Asp Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys
                260                 265                 270

Asp Leu Phe Gly Glu Lys Ile Lys Lys Ser Pro Leu Thr Ile Cys Phe
        275                 280                 285

Pro Glu Tyr Thr Gly Pro Asn Thr Tyr Glu Asp Ala Ala Ala Tyr Ile
290                 295                 300

Gln Ala Gln Phe Glu Ser Lys Asn Arg Ser Pro Asn Lys Glu Ile Tyr
305                 310                 315                 320

Cys His Met Thr Cys Ala Thr Asp Thr Asn Asn Ile Gln Val Val Phe
                325                 330                 335

Asp Ala Val Thr Asp Ile Ile Ile Ala Asn Asn Leu Arg Gly Cys Gly
                340                 345                 350

Leu Tyr


<210> 23
<211> 355
<212> PRT
<213> Homo sapiens

<400> 23
Met Gly Cys Thr Val Ser Ala Glu Asp Lys Ala Ala Ala Glu Arg Ser
  1               5                   10                  15

Lys Met Ile Asp Lys Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Arg
                20                  25                  30

Glu Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
        35                  40                  45

Ile Val Lys Gln Met Lys Ile Ile His Glu Asp Gly Tyr Ser Glu Glu
        50                  55                  60

Glu Cys Arg Gln Tyr Arg Ala Val Val Tyr Ser Asn Thr Ile Gln Ser
```

```
        65                  70                  75                  80

  Ile Met Ala Ile Val Lys Ala Met Gly Asn Leu Gln Ile Asp Phe Ala
                  85                  90                  95

  Asp Pro Ser Arg Ala Asp Asp Ala Arg Gln Leu Phe Ala Leu Ser Cys
                 100             105             110

  Thr Ala Glu Glu Gln Gly Val Leu Pro Asp Asp Leu Ser Gly Val Ile
             115             120             125

  Arg Arg Leu Trp Ala Asp His Gly Val Gln Ala Cys Phe Gly Arg Ser
         130             135             140

  Arg Glu Tyr Gln Leu Asn Asp Ser Ala Ala Tyr Tyr Leu Asn Asp Leu
     145             150             155             160

  Glu Arg Ile Ala Gln Ser Asp Tyr Ile Pro Thr Gln Gln Asp Val Leu
                 165             170             175

  Arg Thr Arg Val Lys Thr Thr Gly Ile Val Glu Thr His Phe Thr Phe
                 180             185             190

  Lys Asp Leu His Phe Lys Met Phe Asp Val Gly Gly Gln Arg Ser Glu
                 195             200             205

  Arg Lys Lys Trp Ile His Cys Phe Glu Gly Val Thr Ala Ile Ile Phe
         210             215             220

  Cys Val Ala Leu Ser Ala Tyr Asp Leu Val Leu Ala Glu Asp Glu Glu
  225             230             235             240

  Met Asn Arg Met His Glu Ser Met Lys Leu Phe Asp Ser Ile Cys Asn
                 245             250             255

  Asn Lys Trp Phe Thr Asp Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys
                 260             265             270

  Asp Leu Phe Glu Glu Lys Ile Thr His Ser Pro Leu Thr Ile Cys Phe
         275             280             285

  Pro Glu Tyr Thr Gly Ala Asn Lys Tyr Asp Glu Ala Ala Ser Tyr Ile
     290             295             300

  Gln Ser Lys Phe Glu Asp Leu Asn Lys Arg Lys Asp Thr Lys Glu Ile
  305             310             315             320

  Tyr Thr His Phe Thr Cys Ala Thr Asp Thr Lys Asn Val Gln Phe Val
                 325             330             335

  Phe Asp Ala Val Thr Asp Val Ile Ile Lys Asn Asn Leu Lys Asp Cys
                 340             345             350

  Gly Leu Phe
         355


  <210> 24
  <211> 350
  <212> PRT
  <213> Homo sapiens
```

```
<400> 24
Met Gly Ala Gly Ala Ser Ala Glu Glu Lys His Ser Arg Glu Leu Glu
1               5                   10                  15

Lys Lys Leu Lys Glu Asp Ala Glu Lys Asp Ala Arg Thr Val Lys Leu
            20              25                  30

Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln
        35              40                  45

Met Lys Ile Ile His Gln Asp Gly Tyr Ser Leu Glu Glu Cys Leu Glu
    50              55                  60

Phe Ile Ala Ile Ile Tyr Gly Asn Thr Leu Gln Ser Ile Leu Ala Ile
65              70                  75                  80

Val Arg Ala Met Thr Thr Leu Asn Ile Gln Tyr Gly Asp Ser Ala Arg
                85                  90                  95

Gln Asp Asp Ala Arg Lys Leu Met His Met Ala Asp Thr Ile Glu Glu
            100             105                 110

Gly Thr Met Pro Lys Glu Met Ser Asp Ile Ile Gln Arg Leu Trp Lys
        115             120                 125

Asp Ser Gly Ile Gln Ala Cys Phe Glu Arg Ala Ser Glu Tyr Gln Leu
    130             135                 140

Asn Asp Ser Ala Gly Tyr Tyr Leu Ser Asp Leu Glu Arg Leu Val Thr
145             150                 155                 160

Pro Gly Tyr Val Pro Thr Glu Gln Asp Val Leu Arg Ser Arg Val Lys
            165                 170                 175

Thr Thr Gly Ile Ile Glu Thr Gln Phe Ser Phe Lys Asp Leu Asn Phe
            180                 185                 190

Arg Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile
        195                 200                 205

His Cys Phe Glu Gly Val Thr Cys Ile Ile Phe Ile Ala Ala Leu Ser
    210             215                 220

Ala Tyr Asp Met Val Leu Val Glu Asp Asp Glu Val Asn Arg Met His
225             230                 235                 240

Glu Ser Leu His Leu Phe Asn Ser Ile Cys Asn His Arg Tyr Phe Ala
            245                 250                 255

Thr Thr Ser Ile Val Leu Phe Leu Asn Lys Lys Asp Val Phe Phe Glu
            260                 265                 270

Lys Ile Lys Lys Ala His Leu Ser Ile Cys Phe Pro Asp Tyr Asp Gly
        275                 280                 285

Pro Asn Thr Tyr Glu Asp Ala Gly Asn Tyr Ile Lys Val Gln Phe Leu
    290                 295                 300

Glu Leu Asn Met Arg Arg Asp Val Lys Glu Ile Tyr Ser His Met Thr
305                 310                 315                 320
```

Cys Ala Thr Asp Thr Gln Asn Val Lys Phe Val Phe Asp Ala Val Thr
              325   330   335

Asp Ile Ile Ile Lys Glu Asn Leu Lys Asp Cys Gly Leu Phe
              340   345   350

<210> 25
<211> 354
<212> PRT
<213> Homo sapiens

<400> 25
Met Gly Ser Gly Ala Ser Ala Glu Asp Lys Glu Leu Ala Lys Arg Ser
 1   5   10   15

Lys Glu Leu Glu Lys Lys Leu Gln Glu Asp Ala Asp Lys Glu Ala Lys
              20   25   30

Thr Val Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr
         35   40   45

Ile Val Lys Gln Met Lys Ile Ile His Gln Asp Gly Tyr Ser Pro Glu
     50   55   60

Glu Cys Leu Glu Phe Lys Ala Ile Ile Tyr Gly Asn Val Leu Gln Ser
 65   70   75   80

Ile Leu Ala Ile Ile Arg Ala Met Thr Thr Leu Gly Ile Asp Tyr Ala
              85   90   95

Glu Pro Ser Cys Ala Asp Asp Gly Arg Gln Leu Asn Asn Leu Ala Asp
              100   105   110

Ser Ile Glu Glu Gly Thr Met Pro Pro Glu Leu Val Glu Val Ile Arg
         115   120   125

Arg Leu Trp Lys Asp Gly Gly Val Gln Ala Cys Phe Glu Arg Ala Ala
     130   135   140

Glu Tyr Gln Leu Asn Asp Ser Ala Ser Tyr Tyr Leu Asn Gln Leu Glu
145   150   155   160

Arg Ile Thr Asp Pro Glu Tyr Leu Pro Ser Glu Gln Asp Val Leu Arg
              165   170   175

Ser Arg Val Lys Thr Thr Gly Ile Ile Glu Thr Lys Phe Ser Val Lys
              180   185   190

Asp Leu Asn Phe Arg Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg
              195   200   205

Lys Lys Trp Ile His Cys Phe Glu Gly Val Thr Cys Ile Ile Phe Cys
     210   215   220

Ala Ala Leu Ser Ala Tyr Asp Met Val Leu Val Glu Asp Asp Glu Val
225   230   235   240

Asn Arg Met His Glu Ser Leu His Leu Phe Asn Ser Ile Cys Asn His
              245   250   255

24

```
Lys Phe Phe Ala Ala Thr Ser Ile Val Leu Phe Leu Asn Lys Lys Asp
            260             265             270

Leu Phe Glu Glu Lys Ile Lys Lys Val His Leu Ser Ile Cys Phe Pro
        275             280             285

Glu Tyr Asp Gly Asn Asn Ser Tyr Asp Asp Ala Gly Asn Tyr Ile Lys
        290             295             300

Ser Gln Phe Leu Asp Leu Asn Met Arg Lys Asp Val Lys Glu Ile Tyr
305             310             315             320

Ser His Met Thr Cys Ala Thr Asp Thr Gln Asn Val Lys Phe Val Phe
                325             330             335

Asp Ala Val Thr Asp Ile Ile Ile Lys Glu Asn Leu Lys Asp Cys Gly
            340             345             350

Leu Phe



<210> 26
<211> 466
<212> PRT
<213> Homo sapiens

<400> 26
Met Val Phe Leu Ser Gly Asn Ala Ser Asp Ser Ser Asn Cys Thr Gln
  1             5               10              15

Pro Pro Ala Pro Val Asn Ile Ser Lys Ala Ile Leu Leu Gly Val Ile
            20              25              30

Leu Gly Gly Leu Ile Leu Phe Gly Val Leu Gly Asn Ile Leu Val Ile
        35              40              45

Leu Ser Val Ala Cys His Arg His Leu His Ser Val Thr His Tyr Tyr
        50              55              60

Ile Val Asn Leu Ala Val Ala Asp Leu Leu Leu Thr Ser Thr Val Leu
 65              70              75              80

Pro Phe Ser Ala Ile Phe Glu Val Leu Gly Tyr Trp Ala Phe Gly Arg
                85              90              95

Val Phe Cys Asn Ile Trp Ala Ala Val Asp Val Leu Cys Cys Thr Ala
            100             105             110

Ser Ile Met Gly Leu Cys Ile Ile Ser Ile Asp Arg Tyr Ile Gly Val
        115             120             125

Ser His Pro Leu Arg Tyr Pro Thr Ile Val Thr Gln Arg Arg Gly Leu
    130             135             140

Met Ala Leu Leu Cys Val Trp Ala Leu Ser Leu Val Ile Ser Ile Gly
145             150             155             160

Pro Leu Phe Gly Trp Arg Gln Pro Ala Pro Glu Asp Glu Thr Ile Cys
                165             170             175
```

```
Gln Ile Asn Glu Glu Pro Gly Tyr Val Leu Phe Ser Ala Leu Gly Ser
        180             185             190

Phe Tyr Leu Pro Leu Ala Ile Ile Leu Val Met Tyr Cys Arg Val Tyr
        195             200             205

Val Val Ala Lys Arg Glu Ser Arg Gly Leu Lys Ser Gly Leu Lys Thr
        210             215             220

Asp Lys Ser Asp Ser Glu Gln Val Thr Leu Arg Ile His Arg Lys Asn
225             230             235             240

Ala Pro Ala Gly Gly Ser Gly Met Ala Ser Ala Lys Thr Lys Thr His
            245             250             255

Phe Ser Val Arg Leu Leu Lys Phe Ser Arg Glu Lys Lys Ala Ala Lys
            260             265             ·270

Thr Leu Gly Ile Val Val Gly Cys Phe Val Leu Cys Trp Leu Pro Phe
        275             280             285

Phe Leu Val Met Pro Ile Gly Ser Phe Phe Pro Asp Phe Lys Pro Ser
        290             295             300

Glu Thr Val Phe Lys Ile Val Phe Trp Leu Gly Tyr Leu Asn Ser Cys
305             310             315             320

Ile Asn Pro Ile Ile Tyr Pro Cys Ser Ser Gln Glu Phe Lys Lys Ala
                325             330             335

Phe Gln Asn Val Leu Arg Ile Gln Cys Leu Cys Arg Lys Gln Ser Ser
            340             345             350

Lys His Ala Leu Gly Tyr Thr Leu His Pro Pro Ser Gln Ala Val Glu
            355             360             365

Gly Gln His Lys Asp Met Val Arg Ile Pro Val Gly Ser Arg Glu Ala
    370             375             380

Phe Tyr Gly Ile Ser Arg Thr Asp Gly Val Cys Glu Trp Lys Phe Phe
385             390             395             400

Ser Ser Met Pro Arg Gly Ser Ala Arg Ile Thr Val Ser Lys Asp Gln
            405             410             415

Ser Ser Cys Thr Thr Ala Arg Val Arg Ser Lys Ser Phe Leu Gln Val
        420             425             430

Cys Cys Cys Val Glu Pro Ser Thr Pro Ser Leu Asp Lys Asn His Gln
        435             440             445

Val Pro Thr Ile Lys Val His Thr Ile Ser Leu Ser Glu Asn Gly Glu
    450             455             460

Glu Val
465


<210> 27
<211> 353
```

```
<212> PRT
<213> Homo sapiens

<400> 27
Met Ala Cys Cys Leu Ser Glu Glu Ala Lys Glu Ala Arg Arg Ile Asn
 1               5                  10                  15

Asp Glu Ile Glu Arg Gln Leu Arg Arg Asp Lys Arg Asp Ala Arg Arg
            20                  25                  30

Glu Leu Lys Leu Leu Leu Leu Gly Thr Gly Glu Ser Gly Lys Ser Thr
            35                  40                  45

Phe Ile Lys Gln Met Arg Ile Ile His Gly Ser Gly Tyr Ser Asp Glu
        50                  55                  60

Asp Lys Arg Gly Phe Thr Lys Leu Val Tyr Gln Asn Ile Phe Thr Ala
65                  70                  75                  80

Met Gln Ala Met Ile Arg Ala Met Asp Thr Leu Lys Ile Pro Tyr Lys
                85                  90                  95

Tyr Glu His Asn Lys Ala His Ala Gln Leu Val Arg Glu Val Asp Val
            100                 105                 110

Glu Lys Val Ser Ala Phe Glu Asn Pro Tyr Val Asp Ala Ile Lys Ser
            115                 120                 125

Leu Trp Asn Asp Pro Gly Ile Gln Glu Cys Tyr Asp Arg Arg Arg Glu
        130                 135                 140

Tyr Gln Leu Ser Asp Ser Thr Lys Tyr Tyr Leu Asn Asp Leu Asp Arg
145                 150                 155                 160

Val Ala Asp Pro Ala Tyr Leu Pro Thr Gln Gln Asp Val Leu Arg Val
                165                 170                 175

Arg Val Pro Thr Thr Gly Ile Ile Glu Tyr Pro Phe Asp Leu Gln Ser
            180                 185                 190

Val Ile Phe Arg Met Val Asp Val Gly Gly Gln Arg Ser Glu Arg Arg
            195                 200                 205

Lys Trp Ile His Cys Phe Glu Asn Val Thr Ser Ile Met Phe Leu Val
    210                 215                 220

Ala Leu Ser Glu Tyr Asp Gln Val Leu Val Glu Ser Asp Asn Glu Asn
225                 230                 235                 240

Arg Met Glu Glu Ser Lys Ala Leu Phe Arg Thr Ile Ile Thr Tyr Pro
                245                 250                 255

Trp Phe Gln Asn Ser Ser Val Ile Leu Phe Leu Asn Lys Lys Asp Leu
            260                 265                 270

Leu Glu Glu Lys Ile Met Tyr Ser His Leu Val Asp Tyr Phe Pro Glu
            275                 280                 285

Tyr Asp Gly Pro Gln Arg Asp Ala Gln Ala Ala Arg Glu Phe Ile Leu
        290                 295                 300
```

27

Lys Met Phe Val Asp Leu Asn Pro Asp Ser Asp Lys Ile Ile Tyr Ser
305   310   315   320

His Phe Thr Cys Ala Thr Asp Thr Glu Asn Ile Arg Phe Val Phe Ala
                325   330   335

Ala Val Lys Asp Thr Ile Leu Gln Leu Asn Leu Lys Glu Tyr Asn Ala
            340   345   350

Val

<210> 28
<211> 359
<212> PRT
<213> Homo sapiens

<400> 28
Met Thr Leu Glu Ser Met Met Ala Cys Cys Leu Ser Asp Glu Val Lys
1   5   10   15

Glu Ser Lys Arg Ile Asn Ala Glu Ile Glu Lys Gln Leu Arg Arg Asp
            20   25   30

Lys Arg Asp Ala Arg Arg Glu Leu Lys Leu Leu Leu Gly Thr Gly
        35   40   45

Glu Ser Gly Lys Ser Thr Phe Ile Lys Gln Met Arg Ile Ile His Gly
        50   55   60

Ala Gly Tyr Ser Glu Glu Asp Lys Arg Gly Phe Thr Lys Leu Val Tyr
65   70   75   80

Gln Asn Ile Phe Thr Ala Met Gln Ala Met Ile Arg Ala Met Glu Thr
                85   90   95

Leu Lys Ile Leu Tyr Lys Tyr Glu Gln Asn Lys Ala Asn Ala Leu Leu
            100   105   110

Ile Arg Glu Val Asp Val Glu Lys Val Thr Thr Phe Glu His Gln Tyr
        115   120   125

Val Ser Ala Ile Lys Thr Leu Trp Glu Asp Pro Gly Ile Gln Glu Cys
    130   135   140

Tyr Asp Arg Arg Arg Glu Tyr Gln Leu Ser Asp Ser Ala Lys Tyr Tyr
145   150   155   160

Leu Thr Asp Val Asp Arg Ile Ala Thr Leu Gly Tyr Leu Pro Thr Gln
                165   170   175

Gln Asp Val Leu Arg Val Arg Val Pro Thr Thr Gly Ile Ile Glu Tyr
            180   185   190

Pro Phe Asp Leu Glu Asn Ile Ile Phe Arg Met Val Asp Val Gly Gly
        195   200   205

Gln Arg Ser Glu Arg Arg Lys Trp Ile His Cys Phe Glu Asn Val Thr
    210   215   220

28

```
Ser Ile Met Phe Leu Val Ala Leu Ser Glu Tyr Asp Gln Val Leu Val
225             230             235             240

Glu Ser Asp Asn Glu Asn Arg Met Glu Glu Ser Lys Ala Leu Phe Arg
            245             250             255

Thr Ile Ile Thr Tyr Pro Trp Phe Gln Asn Ser Ser Val Ile Leu Phe
            260             265             270

Leu Asn Lys Lys Asp Leu Leu Glu Asp Lys Ile Leu Tyr Ser His Leu
        275             280             285

Val Asp Tyr Phe Pro Glu Phe Asp Gly Pro Gln Arg Asp Ala Gln Ala
    290             295             300

Ala Arg Glu Phe Ile Leu Lys Met Phe Val Asp Leu Asn Pro Asp Ser
305             310             315             320

Asp Lys Ile Ile Tyr Ser His Phe Thr Cys Ala Thr Asp Thr Glu Asn
            325             330             335

Ile Arg Phe Val Phe Ala Ala Val Lys Asp Thr Ile Leu Gln Leu Asn
            340             345             350

Leu Lys Glu Tyr Asn Leu Val
            355


<210> 29
<211> 381
<212> PRT
<213> Homo sapiens

<400> 29
Met Ser Gly Val Val Arg Thr Leu Ser Arg Cys Leu Leu Pro Ala Glu
    1               5               10              15

Ala Gly Gly Ala Arg Glu Arg Arg Ala Gly Ser Gly Ala Arg Asp Ala
            20              25              30

Glu Arg Glu Ala Arg Arg Arg Ser Arg Asp Ile Asp Ala Leu Leu Ala
        35              40              45

Arg Glu Arg Arg Ala Val Arg Arg Leu Val Lys Ile Leu Leu Leu Gly
    50              55              60

Ala Gly Glu Ser Gly Lys Ser Thr Phe Leu Lys Gln Met Arg Ile Ile
65              70              75              80

His Gly Arg Glu Phe Asp Gln Lys Ala Leu Leu Glu Phe Arg Asp Thr
                85              90              95

Ile Phe Asp Asn Ile Leu Lys Gly Ser Arg Val Leu Val Asp Ala Arg
            100             105             110

Asp Lys Leu Gly Ile Pro Trp Gln Tyr Ser Glu Asn Glu Lys His Gly
        115             120             125

Met Phe Leu Met Ala Phe Glu Asn Lys Ala Gly Leu Pro Val Glu Pro
    130             135             140
```

```
Ala Thr Phe Gln Leu Tyr Val Pro Ala Leu Ser Ala Leu Trp Arg Asp
145                 150             155                 160

Ser Gly Ile Arg Glu Ala Phe Ser Arg Arg Ser Glu Phe Gln Leu Gly
                165             170             175

Glu Ser Val Lys Tyr Phe Leu Asp Asn Leu Asp Arg Ile Gly Gln Leu
            180             185             190

Asn Tyr Phe Pro Ser Lys Gln Asp Ile Leu Leu Ala Arg Lys Ala Thr
        195             200             205

Lys Gly Ile Val Glu His Asp Phe Val Ile Lys Lys Ile Pro Phe Lys
    210             215             220

Met Val Asp Val Gly Gly Gln Arg Ser Gln Arg Gln Lys Trp Phe Gln
225             230             235             240

Cys Phe Asp Gly Ile Thr Ser Ile Leu Phe Met Val Ser Ser Ser Glu
            245             250             255

Tyr Asp Gln Val Leu Met Glu Asp Arg Arg Thr Asn Arg Leu Val Glu
            260             265             270

Ser Met Asn Ile Phe Glu Thr Ile Val Asn Asn Lys Leu Phe Phe Asn
        275             280             285

Val Ser Ile Ile Leu Phe Leu Asn Lys Met Asp Leu Leu Val Glu Lys
    290             295             300

Val Lys Thr Val Ser Ile Lys Lys His Phe Pro Asp Phe Arg Gly Asp
305             310             315             320

Pro His Gln Leu Glu Asp Val Gln Arg Tyr Leu Val Gln Cys Phe Asp
            325             330             335

Arg Lys Arg Arg Asn Arg Ser Lys Pro Leu Phe His His Phe Thr Thr
            340             345             350

Ala Ile Asp Thr Glu Asn Val Arg Phe Val Phe His Ala Val Lys Asp
        355             360             365

Thr Ile Leu Gln Glu Asn Leu Lys Asp Ile Met Leu Gln
        370             375             380
```

```
<210> 30
<211> 377
<212> PRT
<213> Homo sapiens

<400> 30
Met Ala Asp Phe Leu Pro Ser Arg Ser Val Leu Ser Val Cys Phe Pro
1               5               10              15

Gly Cys Leu Leu Thr Ser Gly Glu Ala Glu Gln Gln Arg Lys Ser Lys
            20              25              30

Glu Ile Asp Lys Cys Leu Ser Arg Glu Lys Thr Tyr Val Lys Arg Leu
        35              40              45
```

```
Val Lys Ile Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr Phe
    50                  55                  60

Leu Lys Gln Met Arg Ile Ile His Gly Gln Asp Phe Asp Gln Arg Ala
    65                  70                  75                  80

Arg Glu Glu Phe Arg Pro Thr Ile Tyr Ser Asn Val Ile Lys Gly Met
                85                  90                  95

Arg Val Leu Val Asp Ala Arg Glu Lys Leu His Ile Pro Trp Gly Asp
            100                 105                 110

Asn Ser Asn Gln Gln His Gly Asp Lys Met Met Ser Phe Asp Thr Arg
            115                 120                 125

Ala Pro Met Ala Ala Gln Gly Met Val Glu Thr Arg Val Phe Leu Gln
    130                 135                 140

Tyr Leu Pro Ala Ile Arg Ala Leu Trp Ala Asp Ser Gly Ile Gln Asn
145                 150                 155                 160

Ala Tyr Asp Arg Arg Arg Glu Phe Gln Leu Gly Glu Ser Val Lys Tyr
                165                 170                 175

Phe Leu Asp Asn Leu Asp Lys Leu Gly Glu Pro Asp Tyr Ile Pro Ser
            180                 185                 190

Gln Gln Asp Ile Leu Leu Ala Arg Arg Pro Thr Lys Gly Ile His Glu
            195                 200                 205

Tyr Asp Phe Glu Ile Lys Asn Val Pro Phe Lys Met Leu Asp Val Gly
    210                 215                 220

Gly Gln Arg Ser Glu Arg Lys Arg Trp Phe Glu Cys Phe Asp Ser Val
225                 230                 235                 240

Thr Ser Ile Leu Phe Leu Val Ser Ser Ser Glu Phe Asp Gln Val Leu
                245                 250                 255

Met Glu Asp Arg Leu Thr Asn Arg Leu Thr Glu Ser Leu Asn Ile Phe
            260                 265                 270

Glu Thr Ile Val Asn Asn Arg Val Phe Ser Asn Val Ser Ile Ile Leu
    275                 280                 285

Phe Leu Asn Lys Thr Asp Leu Leu Glu Glu Lys Val Gln Ile Val Ser
    290                 295                 300

Ile Lys Asp Tyr Phe Leu Glu Phe Glu Gly Asp Pro His Cys Leu Arg
305                 310                 315                 320

Asp Val Gln Lys Phe Leu Val Glu Cys Phe Arg Asn Lys Arg Arg Asp
                325                 330                 335

Gln Gln Gln Lys Pro Leu Tyr His His Phe Thr Thr Ala Ile Asn Thr
                340                 345                 350

Glu Asn Ile Arg Leu Val Phe Arg Asp Val Lys Asp Thr Ile Leu His
            355                 360                 365

Asp Asn Leu Lys Gln Leu Met Leu Gln
```

31

```
         370                    375


<210> 31
<211> 355
<212> PRT
<213> Homo sapiens

<400> 31
Met Ala Gly Cys Cys Cys Leu Ser Ala Glu Glu Lys Glu Ser Gln Arg
 1               5                  10                  15

Ile Ser Ala Glu Ile Glu Arg Gln Leu Arg Arg Asp Lys Lys Asp Ala
            20                  25                  30

Arg Arg Glu Leu Lys Leu Leu Leu Leu Gly Thr Gly Glu Ser Gly Lys
         35                  40                  45

Ser Thr Phe Ile Lys Gln Met Arg Ile Ile His Gly Ser Gly Tyr Ser
      50                  55                  60

Asp Glu Asp Arg Lys Gly Phe Thr Lys Leu Val Tyr Gln Asn Ile Phe
 65                  70                  75                  80

Thr Ala Met Gln Ala Met Ile Arg Ala Met Asp Thr Leu Arg Ile Gln
                85                  90                  95

Tyr Val Cys Glu Gln Asn Lys Glu Asn Ala Gln Ile Ile Arg Glu Val
            100                 105                 110

Glu Val Asp Lys Val Ser Met Leu Ser Arg Glu Gln Val Glu Ala Ile
         115                 120                 125

Lys Gln Leu Trp Gln Asp Pro Gly Ile Gln Glu Cys Tyr Asp Arg Arg
      130                 135                 140

Arg Glu Tyr Gln Leu Ser Asp Ser Ala Lys Tyr Tyr Leu Thr Asp Ile
145                 150                 155                 160

Asp Arg Ile Ala Thr Pro Ser Phe Val Pro Thr Gln Gln Asp Val Leu
                165                 170                 175

Arg Val Arg Val Pro Thr Thr Gly Ile Ile Glu Tyr Pro Phe Asp Leu
            180                 185                 190

Glu Asn Ile Ile Phe Arg Met Val Asp Val Gly Gly Gln Arg Ser Glu
         195                 200                 205

Arg Arg Lys Trp Ile His Cys Phe Glu Ser Val Thr Ser Ile Ile Phe
      210                 215                 220

Leu Val Ala Leu Ser Glu Tyr Asp Gln Val Leu Ala Glu Cys Asp Asn
225                 230                 235                 240

Glu Asn Arg Met Glu Glu Ser Lys Ala Leu Phe Lys Thr Ile Ile Thr
                245                 250                 255

Tyr Pro Trp Phe Leu Asn Ser Ser Val Ile Leu Phe Leu Asn Lys Lys
            260                 265                 270

Asp Leu Leu Glu Glu Lys Ile Met Tyr Ser His Leu Ile Ser Tyr Phe
```

```
            275               280               285
Pro Glu Tyr Thr Gly Pro Lys Gln Asp Val Arg Ala Ala Arg Asp Phe
    290               295           300
Ile Leu Lys Leu Tyr Gln Asp Gln Asn Pro Asp Lys Glu Lys Val Ile
305               310           315               320
Tyr Ser His Phe Thr Cys Ala Thr Asp Thr Asp Asn Ile Arg Phe Val
            325               330               335
Phe Ala Ala Val Lys Asp Thr Ile Leu Gln Leu Asn Leu Arg Glu Phe
            340               345           350
Asn Leu Val
    355
```

```
<210> 32
<211> 374
<212> PRT
<213> Homo sapiens

<400> 32
Met Ala Arg Ser Leu Thr Trp Arg Cys Cys Pro Trp Cys Leu Thr Glu
  1               5               10               15
Asp Glu Lys Ala Ala Ala Arg Val Asp Gln Glu Ile Asn Arg Ile Leu
            20               25               30
Leu Glu Gln Lys Lys Gln Asp Arg Gly Glu Leu Lys Leu Leu Leu Leu
        35               40               45
Gly Pro Gly Glu Ser Gly Lys Ser Thr Phe Ile Lys Gln Met Arg Ile
    50               55               60
Ile His Gly Ala Gly Tyr Ser Glu Glu Glu Arg Lys Gly Phe Arg Pro
65               70               75               80
Leu Val Tyr Gln Asn Ile Phe Val Ser Met Arg Ala Met Ile Glu Ala
            85               90               95
Met Glu Arg Leu Gln Ile Pro Phe Ser Arg Pro Glu Ser Lys His His
            100               105               110
Ala Ser Leu Val Met Ser Gln Asp Pro Tyr Lys Val Thr Thr Phe Glu
        115               120               125
Lys Arg Tyr Ala Ala Ala Met Gln Trp Leu Trp Arg Asp Ala Gly Ile
    130               135               140
Arg Ala Cys Tyr Glu Arg Arg Arg Glu Phe His Leu Leu Asp Ser Ala
145               150               155               160
Val Tyr Tyr Leu Ser His Leu Glu Arg Ile Thr Glu Glu Gly Tyr Val
            165               170               175
Pro Thr Ala Gln Asp Val Leu Arg Ser Arg Met Pro Thr Thr Gly Ile
        180               185               190
Asn Glu Tyr Cys Phe Ser Val Gln Lys Thr Asn Leu Arg Ile Val Asp
```

33

```
        195                 200                 205
Val Gly Gly Gln Lys Ser Glu Arg Lys Lys Trp Ile His Cys Phe Glu
    210                 215                 220

Asn Val Ile Ala Leu Ile Tyr Leu Ala Ser Leu Ser Glu Tyr Asp Gln
225                 230                 235                 240

Cys Leu Glu Glu Asn Asn Gln Glu Asn Arg Met Lys Glu Ser Leu Ala
            245                 250                 255

Leu Phe Gly Thr Ile Leu Glu Leu Pro Trp Phe Lys Ser Thr Ser Val
            260                 265                 270

Ile Leu Phe Leu Asn Lys Thr Asp Ile Leu Glu Glu Lys Ile Pro Thr
            275                 280                 285

Ser His Leu Ala Thr Tyr Phe Pro Ser Phe Gln Gly Pro Lys Gln Asp
    290                 295                 300

Ala Glu Ala Ala Lys Arg Phe Ile Leu Asp Met Tyr Thr Arg Met Tyr
305                 310                 315                 320

Thr Gly Cys Val Asp Gly Pro Glu Gly Ser Lys Lys Gly Ala Arg Ser
            325                 330                 335

Arg Arg Leu Phe Ser His Tyr Thr Cys Ala Thr Asp Thr Gln Asn Ile
            340                 345                 350

Arg Lys Val Phe Lys Asp Val Arg Asp Ser Val Leu Ala Arg Tyr Leu
            355                 360                 365

Asp Glu Ile Asn Leu Leu
    370


<210> 33
<211> 157
<212> PRT
<213> Homo sapiens

<400> 33
Met Phe Asp Val Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile His
  1                 5                  10                  15

Cys Phe Glu Gly Val Thr Cys Ile Ile Phe Cys Ala Ala Leu Ser Ala
                20                  25                  30

Tyr Asp Met Val Leu Val Glu Asp Glu Glu Val Asn Arg Met His Glu
            35                  40                  45

Ser Leu His Leu Phe Asn Ser Ile Cys Asn His Lys Tyr Phe Ser Thr
        50                  55                  60

Thr Ser Ile Val Leu Phe Leu Asn Lys Lys Asp Ile Phe Gln Glu Lys
65                  70                  75                  80

Val Thr Lys Val His Leu Ser Ile Cys Phe Pro Glu Tyr Thr Gly Pro
                85                  90                  95

Asn Thr Phe Glu Asp Ala Gly Asn Tyr Ile Lys Asn Gln Phe Leu Asp
```

34

```
                100                    105                    110
Leu Asn Leu Lys Lys Glu Asp Lys Glu Ile Tyr Ser His Met Thr Cys
        115                 120                 125

Ala Thr Asp Thr Gln Asn Val Lys Phe Val Phe Asp Ala Val Thr Asp
        130                 135                 140

Ile Ile Ile Lys Glu Asn Leu Lys Asp Cys Gly Leu Phe
145                 150                 155
```

```
<210> 34
<211> 75
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
     cAMP-Hammerhead RNA sequence

<400> 34
gggcgacccu gaugagccug uggaaacaga cguggcacau gacuacgucg aaacggugaa 60
agccguaggu ugccc                                                  75


<210> 35
<211> 75
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
     cGMP-Hammerhead RNA sequence

<400> 35
gggcgacccu gaugagcccu gcgaugcaga aaggugcuga cgacacaucg aaacggugaa 60
agccguaggu ugccc                                                  75


<210> 36
<211> 164
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Template for
     randomized sequence

<220>
<221> misc_feature
<222> (23)
<223> Wherein n is t or a or c or g.

<220>
<221> misc_feature
<222> (36)
<223> Wherein n is a or t or a or c or g.

<220>
<221> misc_feature
```

<220>
<221> misc_feature
<222> (42)
<223> Wherein n is t or a or c or g.

<220>
<221> misc_feature
<222> (55)..(56)
<223> Wherein n is t or a or c or g.

<220>
<221> misc_feature
<222> (58)
<223> Wherein n is t or a or c or g.

<220>
<221> misc_feature
<222> (63)
<223> Wherein n is t or a or c or g.

<220>
<221> misc_feature
<222> (69)..(118)
<223> Wherein n is an a or u or c or g and some may be
      missing.

<400> 36
ggagttacct aacaagaaac agngaagtca accagngaaa cncacggaga cgtgnnanat 60
tanctggtnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnngg 120
acctactgag ctgacagtcc tgtttgatgc ataccgagta agtg   164

<210> 37
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-D02
      ERK specific nucleic acid sensor molecule

<400> 37
ggacuucggc gaaagccguu cgacccuacu cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugaggg cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 38
<211> 132
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-D05
      ERK specific nucleic acid sensor molecule

<400> 38
ggacuucggc gaaagccguu cgaccuucuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaguaa gcuuagacag gagguuaggu gcgucaaugc 120
ugcaaguuac ug   132

<210> 39

```
<211> 133
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-E01

<400> 39
ggacuucggc gaaagccguu cgacccgcag cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugcggu ggcuuagaca ggagguuagg ugcgucaaug 120
cugcaaguua cug                                                    133


<210> 40
<211> 95
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cCMP nucleic
      acid sensor molecule

<400> 40
ggacccugau gagccuuuag ggccaagugu ggugaaagac acacgucgaa acggugaaag 60
ccguaggucc uugcgugguu cuguucccuu cuucg                            95


<210> 41
<211> 94
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cAMP nucleic
      acid sensor molecule

<400> 41
ggacccugau gagccugugg aaacagacgu ggcacaugac uacgucgaaa cggugaaagc 60
cguagguccu ugcgugguuc uguucccuuc uucg                             94


<210> 42
<211> 94
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cGMP nucleic
      acid sensor molecule

<400> 42
ggacccugau gagccuugcg augcaaaaag gugcugacga cacaucgaaa cggugaaagc 60
cguagguccu ugcgugguuc uguucccuuc uucg                             94


<210> 43
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
```

```
<223> Description of Artificial Sequence: Capture oligo

<400> 43
acgcaccaag acaagggaag aagc                                        24


<210> 44
<211> 133
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-A02
      ppERK nucleic acid sensor molecule

<400> 44
ggacuucggc gaaagccguu cgaccgguug cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaauggaac cgcuuagaca ggagguuagg ugcgucaaug 120
cugcaaguua cug                                                    133


<210> 45
<211> 133
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW-45-33-B04
      ppERK nucleic acid sensor molecule

<400> 45
ggacuucggc gaaagccguu cgaccgguug cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugugau cgcuuagaca ggagguuagg ugcgucaaug 120
cugcaaguua cug                                                    133


<210> 46
<211> 96
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nucleic acid
      sensor precursor molecule

<400> 46
ggcgugaccu gaugagucac gcagacgcua gcgaauuggu uccucaaagg gggaaagcgu 60
uauuaagaaa ccaaaaugug uuuacgaaac guuccc                           96


<210> 47
<211> 108
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nucleic acid
      sensor precursor molecule

<400> 47
ggacuucggu ccagugcucg ugcacuaggc cguucgacca ugauaccagc aucgucuuga 60
```

ugcccuuggc agcaucuuag acaggagguu aggugccucg ugaugucc   108

<210> 48
<211> 79
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nucleic acid
      sensor precursor molecule

<400> 48
gggcgacccu gaugagccug gauaccaagc cgaaaggccc uuggcaguua gacgaaacgg 60
ugaaagccgu agguugccc   79

<210> 49
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nucleic acid
      sensor molecule

<220>
<221> misc_feature
<222> (1)
<223> First signaling moiety.

<220>
<221> misc_feature
<222> (34)
<223> Second signaling moiety.

<400> 49
nauaccaagc cgaaaggccc uuggcagugg uaun   34

<210> 50
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Thrombin
      nucleic acid sensor molecule

<400> 50
ccaaccggtt ggtgtggttg g   21

<210> 51
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Target
      molecule

```
<400> 51
gcgactggac atcacgag                                        18


<210> 52
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKA peptide

<400> 52
Leu Arg Ala Ser Leu Gly
 1               5


<210> 53
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC peptide

<400> 53
Ala Ala Lys Ile Gln Ala Ser Phe Arg Gly His Met Ala Arg Lys Lys
 1               5                   10                  15


<210> 54
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cdc2 peptide

<400> 54
Pro Lys Thr Pro Lys Lys Ala Lys Lys Leu
 1               5                   10


<210> 55
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: DNA-PK peptide

<400> 55
Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys
 1               5                   10                  15


<210> 56
<211> 10
<212> PRT
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence: CK-1 peptide

<400> 56
Asp Asp Asp Glu Glu Ser Ile Thr Arg Arg
 1   5   10

<210> 57
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CK-2 peptide

<400> 57
Arg Arg Arg Glu Glu Glu Thr Glu Glu Glu
 1   5   10

<210> 58
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cam KII
       peptide

<400> 58
Lys Lys Ala Leu Arg Arg Gln Glu Thr Val Asp Ala Leu
 1   5   10

<210> 59
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: p38 peptide

<400> 59
Ser Thr Lys Val Pro Gln Thr Pro Leu His Thr Ser Arg Val
 1   5   10

<210> 60
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKA peptide

<400> 60
Arg Arg Arg Arg Ser Ile Ile Phe Ile
 1   5

<210> 61
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKA alpha
      peptide

<400> 61
Arg Arg Arg Arg Arg Lys Gly Ser Phe Arg Arg Lys Ala
 1   5   10

<210> 62
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC beta I, II
      peptide

<400> 62
Arg Lys Leu Lys Arg Lys Gly Ser Phe Arg Arg Lys Ala
 1   5   10

<210> 63
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC gammaI
      peptide

<400> 63
Arg Arg Arg Arg Arg Lys Gly Ser Phe Lys Lys Phe Ala
 1   5   10

<210> 64
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC delta
      peptide

<400> 64
Ala Ala Arg Lys Arg Lys Gly Ser Phe Phe Tyr Gly Gly
 1   5   10

<210> 65
<211> 13
<212> PRT
<213> Artificial Sequence

```
<220>
<223> Description of Artificial Sequence: PKC epsilon
      peptide

<220>
<221> VARIANT
<222> (3)
<223> Wherein Xaa is any amino acid.

<400> 65
Tyr Tyr Xaa Lys Arg Lys Met Ser Phe Phe Glu Phe Asp
  1           5                   10


<210> 66
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC eta
      peptide

<220>
<221> VARIANT
<222> (12)
<223> Wherein Xaa is any amino acid.

<400> 66
Ala Arg Leu Arg Arg Arg Arg Ser Phe Arg Arg Xaa Arg
  1           5                   10


<210> 67
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC zeta
      peptide

<400> 67
Arg Arg Phe Lys Arg Gln Gly Ser Phe Phe Tyr Phe Phe
  1           5                   10


<210> 68
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PKC mu peptide

<400> 68
Ala Ala Leu Val Arg Gln Met Ser Val Ala Phe Phe Phe
  1           5                   10
```

<210> 69
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cam KII
      peptide

<400> 69
Lys Arg Gln Gln Ser Phe Asp Leu Phe
  1   5

<210> 70
<211> 9   .
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: phosphorylase
      kinaseI peptide

<400> 70
Phe Arg Met Met Ser Phe Phe Leu Phe
  1   5

<210> 71
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: SLK1 peptide

<400> 71
Arg Arg Phe Gly Ser Leu Arg Arg Phe
  1   5

<210> 72
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: SPRK2   peptide

<400> 72
Arg Arg Arg His Ser Arg Arg Arg Arg
  1   5

<210> 73
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: AKT/PKB

44

peptide

<220>
<221> VARIANT
<222> (4)
<223> Wherein Xaa is any amino acid.

<400> 73
Arg Lys Arg Xaa Arg Thr Tyr Ser Phe Gly
 1   5   10

<210> 74
<211> 28
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector
      dependent release factor

<400> 74
guccuguuug augcauaccg aguaagug .   28

<210> 75
<211> 146
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Substrate oligonucleotide

<220>
<223> Description of Artificial Sequence: ERK specific
      ligase nucleic acid sensor molecule

<400> 75
uaagcucuac aggaaccugg uuuucgcgug gauuggagga cagauucugu gaccgccgug 60
caucggcgaa agccuuuagg aggaaucgca ccagcuugcc gaaagcggcu ucaggucacg 120
tccagtagac tagcattcca gcgtac   146

<210> 76
<211> 145
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Ligase nucleic
      acid sensor molecule

<400> 76
catgcgacct tacgatcaga tgacctgcac uggacuucgg cgaaagccgu ucgaccacgc 60
uaaggaggau uuccgaaagc ggcuacgugc cgccaguguc uuagacagga gguuaggugc 120
gcuuuggucc aaggacaucu cgaau   145

<210> 77
<211> 25

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Capture oligo

<400> 77
attcgagatg uccttggacc aaagc   25

<210> 78
<211> 121
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Hammerhead
      (endonuclease) nucleic acid sensor molecule

<400> 78
cucgagagcg auggcaaagc ugcaucagua cacggugcag acaaaggugu ccgaguaguc 60
ccagcgaaag cguuggaugc cgauaauagg uuuuuucccg uaacguucgc ugaccuguag 120
g   121

<210> 79
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Capture oligo

<400> 79
cctacaggtc agcgaacgtt acgggt   26

<210> 80
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK2 activated
      nucleic acid sensor

<220>
<221> misc_feature
<222> (17)..(20)
<223> Wherein n is a or u or c or g or nothing.

<220>
<221> misc_feature
<222> (58)..(61)
<223> Wherein n is a or u or c or g or nothing.

<400> 80
gggcgacccu gaugagnnnn cuaaggagga uuuccgaaag cggcuacggu ccgccagnnn 60
ncgaaacggu gaaagccgua gguugccc   88

46

<210> 81
<211> 110
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
     sensitive cis-hammerhead nucleic acid sensor
     molecule construct 6

<400> 81
gggcgacccu gaugagucac gcagacgcua gcgaauuggu uccucgaaag gggaaagcgu 60
uauuaagaaa ccaaaaugug uuacgaaacg gugaaaggcc guagguagcc   110

<210> 82
<211> 108
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
     sensitive cis-hammerhead nucleic acid sensor
     molecule construct 7

<400> 82
gggcgacccu gaugagucac gagacgcuag cgaauugguu ccucgaaagg ggaaagcguu 60
auuaagaaac caaaauuguu acgaaacggu gaaaggccgu agguagcc   108

<210> 83
<211> 108
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
     sensitive cis-hammerhead nucleic acid sensor
     molecule construct 8

<400> 83
gggcgacccu gaugagcacg cagacgcuag cgaauugguu ccucgaaagg ggaaagcguu 60
auuaagaaac caaaaugugu ucgaaacggu gaaaggccgu agguagcc   108

<210> 84
<211> 108
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
     sensitive cis-hammerhead nucleic acid sensor
     molecule construct 9

<400> 84
gggcgacccu gaugagucag cagacgcuag cgaauugguu ccucgaaagg ggaaagcguu 60
auuaagaaac caaaauguuu acgaaacggu gaaaggccgu agguagcc   108

<210> 85

```
<211> 110
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      sensitive cis-hammerhead nucleic acid sensor
      molecule construct 10

<400> 85
gggcgacccu gaugagucac gcagacgcua gcgaauuggu uccucgaaag gggaaagcgu 60
uauuaagaaa ccaaaaugug uugcgaaacg gugaaaggcc guagguagcc            110


<210> 86
<211> 104
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      sensitive cis-hammerhead nucleic acid sensor
      molecule construct 11

<400> 86
gggcgacccu gaugagugca gacgcuagcg aauugguucc ucgaaagggg aaagcguuau 60
uaagaaacca aaaugugcga acggugaaa ggccguaggu agcc                   104


<210> 87
<211> 110
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      sensitive cis-hammerhead nucleic acid sensor
      molecule construct 12

<400> 87
gggcgacccu gaugagccuu gcagacgcua gcgaauuggu uccucgaaag gggaaagcgu 60
uauuaagaaa ccaaaaugua cgucgaaacg gugaaaggcc guagguagcc            110


<210> 88
<211> 110
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      sensitive cis-hammerhead nucleic acid sensor
      molecule construct 13

<400> 88
gggcgacccu gaugagucug gcagacgcua gcgaauuggu uccucgaaag gggaaagcgu 60
uauuaagaaa ccaaaauguc auacgaaacg gugaaaggcc guagguagcc            110


<210> 89
<211> 110
```

48

<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppBRK
     sensitive cis-hammerhead nucleic acid sensor
     molecule construct 14

<400> 89
gggcgacccu gaugagucug gcagacgcua gcgaauuggu uccucgaaag gggaaagcgu 60
uauuaagaaa ccaaaauguc uuacgaaacg gugaaaggcc guagguagcc   110

<210> 90
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-F08
     ERK specific nucleic acid sensor molecule

<400> 90
ggacuucggc gaaagccguu cgaccuacuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaguag cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 91
<211> 132
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-H08
     ERK specific nucleic acid sensor molecule

<400> 91
ggacuucggc gaaagccguu cgaccccugu cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugacaa gcuuagacag gagguuaggu gcgucaaugc 120
ugcaaguuac ug   132

<210> 92
<211> 132
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-H09
     ERK specific nucleic acid sensor molecule

<400> 92
ggacuucggc gaaagccguu cgaccucgcu cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugagcg acuuagacag gagguuaggu gcgucaaugc 120
ugcaaguuac ug   132

<210> 93
<211> 131
<212> RNA

49

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-A10
      ERK specific nucleic acid sensor molecule

<400> 93
ggacuucggc gaaagccguu cgaccaacuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaauggguug cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 94
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-F09
      ERK specific nucleic acid sensor molecule

<400> 94
ggacuucggc gaaagccguu cgaccuguuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaacag cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 95
<211> 132
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-G08
      ERK specific nucleic acid sensor molecule

<400> 95
ggacuucggc gaaagccguu cgacccuucu cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugagaa gcuuagacag gagguuaggu gcgucaaugc 120
ugcaaguuac ug   132

<210> 96
<211> 134
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-G02
      ppERK specific nucleic acid sensor molecule

<400> 96
ggacuucggc gaaagccguu cgaccauuug ucagacgcua gcgaauuggu uccucgaaag 60
ggaaagcguu auuaagaaac caaaauggca gaucuuagac aggagguuag gugcgucaau 120
gcugcaaguu acug   134

<210> 97
<211> 134
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-G03
      ppERK specific nucleic acid sensor molecule

<400> 97
ggacuucggc gaaagccguu cgaccuuuag ucagacgcua gcgaauuggu uccucgaaag 60
ggaaagcguu auuaagaaac caaaaugauu agacuuagac aggagguuag gugcgucaau 120
gcugcaaguu acug   134

<210> 98
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-H03
      ppERK specific nucleic acid sensor molecule

<400> 98
ggacuucggc gaaagccguu cgacccgcuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaucgg cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 99
<211> 130
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-H1
      ppERK specific nucleic acid sensor molecule

<400> 99
ggacuucggc gaaagccguu cgaccguugc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugcaauc uuagacagga gguuaggugc gucaaugcug 120
caaguuacug   130

<210> 100
<211> 130
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-B05
      ppERK specific nucleic acid sensor molecule

<400> 100
ggacuucggc gaaagccguu cgaccuuagc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugcuaac uuagacagga gguuaggugc gucaaugcug 120
caaguuacug   130

<210> 101
<211> 98
<212> RNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: cGMP specific
      hammerhead nucleic acid sensor molecule

<400> 101
ggauguccag ucgcuugcaa ugcccuuuua gacccugaug agccugugga aacagacgug 60
gcacaugacu acgucgaaac ggugaaagcc guaggucu                        98


<210> 102
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Capture oligo

<400> 102
tgggcattgc aagcgactgg acatcc                                      26


<210> 103
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cAMP specific
      nucleic acid sensor molecule

<400> 103
gggacccuga ugagccugug gaaacagacg uggcacauga cuacgucgaa acggugaaag 60
ccguaggucc                                                       70


<210> 104
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule: nucleic
      acid sensor molecule ligase

<220>
<223> Description of Artificial Sequence: Capture oligo

<400> 104
cgaaaccagg ttccgtagag ctta                                        24


<210> 105
<211> 81
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: C.lys.L1.A
      lysozyme sensitive ligase nucleic acid sensor

<400> 105
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ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uagcgagagu g   81

<210> 106
<211> 87
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: C.lys.L1.B
     ligase nucleic acid sensor molecule

<400> 106
ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uaggugcgag agcacug   87

<210> 107
<211> 98
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: C.lys.L1.C
     ligase nucleic acid sensor molecule

<400> 107
ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uaaggugcga gccgagaggc ucgcacug   98

<210> 108
<211> 119
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK dependent
     ligase nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (33)..(36)
<223> Wherein n is a or u or t or g or nothing.

<220>
<221> misc_feature
<222> (70)..(73)
<223> Wherein n is a or u or c or g or nothing.

<400> 108
ugcacuggac uucggcgaaa gccguucgac cagcuaagga ggauuuccga aagcggcuac 60
gugccgccag cucuuagaca ggagguuagg ugccguccga cugaucucgg aguuaaacg 119

<210> 109
<211> 121
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
        ligase nucleic acid sensor moleucle construct 17

<400> 109
ugcacuggac uucggcgaaa gccguucgac cacgcuaagg aggauuuccg aaagcggcua 60
cgugccgcca gugucuuaga caggagguua ggugccgucc gacugaucuc ggaguuaaac 120
g   .   121

<210> 110
<211> 119
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
        ligase nucleic acid sensor moleucle construct 18

<400> 110
ugcacuggac uucggcgaaa gccguucgac cagcuaagga ggauuuccga aagcggcuac 60
gugccgccag uucuuagaca ggagguuagg ugccguccga cugaucucgg aguuaaacg 119

<210> 111
<211> 119
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
        ligase nucleic acid sensor moleucle construct 19

<400> 111
ugcacuggac uucggcgaaa gccguucgac cagcuaagga ggauuuccga aagcggcuac 60
gugccgccag cucuuagaca ggagguuagg ugccguccga cugaucucgg aguuaaacg 119

<210> 112
<211> 121
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
        ligase nucleic acid sensor moleucle construct 20

<400> 112
ugcacuggac uucggcgaaa gccguucgac cacgguaagg aggauuuccg aaagcggcua 60
cgugccgcca uugucuuaga caggagguua ggugccgucc gacugaucuc ggaguuaaac 120
g   121

<210> 113
<211> 121
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
        ligase nucleic acid sensor moleucle construct 21

54

<400> 113
ugcacuggac uucggcgaaa gccguucgac caccuuaagg aggauuuccg aaagcggcua 60
cgugccgcca cguucuuaga caggagguua ggugccgucc gacugaucuc ggaguuaaac 120
g   121

<210> 114
<211> 119
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
     ligase nucleic acid sensor moleucle construct 22

<400> 114
ugcacuggac uucggcgaaa gccguucgac ccgcuaagga ggauuuccga aagcggcuac 60
gugccgccag ugcuuagaca ggagguuagg ugccguccga cugaucucgg aguuaaacg 119

<210> 115
<211> 117
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
     ligase nucleic acid sensor moleucle construct 23

<400> 115
ugcacuggac uucggcgaaa gccguucgac cgcuaaggag gauuuccgaa agcggcuacg 60
ugccgccagu cuuagacagg agguuaggug ccguccgacu gaucucggag uuaaacg   117

<210> 116
<211> 117
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
     ligase nucleic acid sensor moleucle construct 24

<400> 116
ugcacuggac uucggcgaaa gccguucgac cgcuaaggag gauuuccgaa agcggcuacg 60
ugccgccagc cuuagacagg agguuaggug ccguccgacu gaucucggag uuaaacg   117

<210> 117
<211> 119
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK depedent
     ligase nucleic acid sensor moleucle construct 26

<400> 117
ugcacuggac uucggcgaaa gccguucgac cccuuaagga ggauuuccga aagcggcuac 60
gugccgccac gucuuagaca ggagguuagg ugccguccga cugaucucgg aguuaaacg 119

<210> 118
<211> 118
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 3-piece ERK
      dependent ligase

<220>
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<222> (32)..(35)
<223> Wherein n is a or u or c or g or nothing.

<220>
<221> misc_feature
<222> (68)..(71)
<223> Wherein n is a or u or c or g or nothing.

<400> 118
ugcacuggac uucggcgaaa gccguucgac cnnnnaagga ggauuuccga aagcggcuac   60
ggccgccnnn ncuuagacag gagguuaggu gccguccgac ugaucucgga guuaaacg   118

<210> 119
<211> 95
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 1-piece ERK
      dependent kinase

<400> 119
ggacuucggc gaaagccguu cgaccagcua aggaggauuu ccgaaagcgg cuacggccgc   60
cagcucuuag acaggagguu aggugcgaga gcacu   95

<210> 120
<211> 96
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      template

<220>
<221> misc_feature
<222> (1)..(13)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (83)..(96)
<223> Wherein n is an a or u or c or g or nothing

<400> 120

nnnnnnnnnn nnngucacgc agacgcuagc gaauugguuc cucgaaaggg gaaagcguua 60
uuaagaaacc aaaauguguu acnnnnnnnn nnnnnn   96

<210> 121
<211> 139
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      template

<400> 121
ggacttcggc gaaagccgtt cgaccacgct cagacgctag cgaattggtt cctcgaaagg 60
ggaaagcgtt attaagaaac caaaatgagt gtcttagaca ggaggttagg tgcggctttg 120
gtccaaggac atctcgaat   139

<210> 122
<211> 137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.L

<400> 122
ggacttcggc gaaagccgtt cgaccagctc agacgctagc gaattggttc ctcgaaaggg 60
gaaagcgtta ttaagaaacc aaaatgagtt cttagacagg aggttaggtg cggctttggt 120
ccaaggacat ctcgaat   137

<210> 123
<211> 139
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.M

<400> 123
ggacttcggc gaaagccgtt cgaccacggt cagacgctag cgaattggtt cctcgaaagg 60
ggaaagcgtt attaagaaac caaaatgatt gtcttagaca ggaggttagg tgcggctttg 120
gtccaaggac atctcgaat   139

<210> 124
<211> 139
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.N

<400> 124
ggacttcggc gaaagccgtt cgaccacctt cagacgctag cgaattggtt cctcgaaagg 60
ggaaagcgtt attaagaaac caaaatgacg ttcttagaca ggaggttagg tgcggctttg 120
gtccaaggac atctcgaat   139

<210> 125
<211> 137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.O

<400> 125
ggacttcggc gaaagccgtt cgaccagctc agacgctagc gaattggttc ctcgaaaggg 60
gaaagcgtta ttaagaaacc aaaatgagct cttagacagg aggttaggtg cggctttggt 120
ccaaggacat ctcgaat   137

<210> 126
<211> 137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.P

<400> 126
ggacttcggc gaaagccgtt cgacccgctc agacgctagc gaattggttc ctcgaaaggg 60
gaaagcgtta ttaagaaacc aaaatgagtg cttagacagg aggttaggtg cggctttggt 120
ccaaggacat ctcgaat   137

<210> 127
<211> 135
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      dependent ligase nucleic acid sensor molecule
      construct TK.16.118.Q

<400> 127
ggacttcggc gaaagccgtt cgaccgctca gacgctagcg aattggttcc tcgaaagggg 60
aaagcgttat taagaaacca aaatgagtct tagacaggag gttaggtgcg gctttggtcc 120
aaggacatct cgaat   135

<210> 128
<211> 137
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: ppERK
       dependent ligase nucleic acid sensor molecule
       construct TK.16.118.R

<400> 128
ggacttcggc gaaagccgtt cgacccggtc agacgctagc gaattggttc ctcgaaaggg 60
gaaagcgtta ttaagaaacc aaaatgattg cttagacagg aggttaggtg cggctttggt 120
ccaaggacat ctcgaat   137

<210> 129
<211> 137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
       dependent ligase nucleic acid sensor molecule
       construct TK.16.118.S

<400> 129
ggacttcggc gaaagccgtt cgacccttc agacgctagc gaattggttc ctcgaaaggg 60
gaaagcgtta ttaagaaacc aaaatgacgt cttagacagg aggttaggtg cggctttggt 120
ccaaggacat ctcgaat   137

<210> 130
<211> 135
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
       dependent ligase nucleic acid sensor molecule
       construct TK.16.118.T

<400> 130
ggacttcggc gaaagccgtt cgaccgctca gacgctagcg aattggttcc tcgaaagggg 60
aaagcgttat taagaaacca aaatgagcct tagacaggag gttaggtgcg gctttggtcc 120
aaggacatct cgaat   135

<210> 131
<211> 132
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-C08
       BRK specific nucleic acid sensor molecule

<400> 131
ggacuucggc gaaagccguu cgaccccucu cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugaggg gcuuagacag gagguuaggu gcgucaaugc 120
ugcaaguuac ug   132

<210> 132
<211> 131
<212> RNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: CW45-33-C09
      ERK specific nucleic acid sensor molecule

<400> 132
ggacuucggc gaaagccguu cgaccugauc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaacag cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g                                                      131


<210> 133
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-D09
      ERK specific nucleic acid sensor molecule

<400> 133
ggacuucggc gaaagccguu cgaccuuuuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugaaaag cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g                                                      131


<210> 134
<211> 108
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      Sensitive nucleic acid sensor

<400> 134
ggcaaccuac ggcctttcac cgtttcgtaa cacattttgg tttcttaata acgctttccc 60
ctttcgagga accaattcgc tagcgtctgc gtgactcatc agggtcgc              108


<210> 135
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cGMP specific
      nucleic acid sensor molecule

<400> 135
ggauaauagc cguagguugc gaaagcgacc cugaugagcc cugcgaugca gaaaggugcu 60
gacgacacau cgaaacggua gcgagagcuc                                   90


<210> 136
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cCMP specific
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nucleic acid sensor molecule

<400> 136
ggauaauagc cguagguugc gaaagcgacc cugaugaccu guggaaacag acguggcaca 60
ugacuacgug aaacgguagc gagagcuc   88

<210> 137
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cAMP specific
      nucleic acid sensor molecule

<400> 137
ggauaauagc cguagguugc gaaagcgacc cugaugaccu ugcgaugcaa aaaggugcug 60
acgacacaug aaacgguagc gagagcuc   88

<210> 138
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cCMP specific
      nucleic acid sensor molecule

<400> 138
gggacccuga ugagccuuua gggccaagug uggugaaaga cacacgucga aacggugaaa 60
gccguagguc c   71

<210> 139
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cGMP specific
      nucleic acid sensor molecule

<400> 139
gggacccuga ugagccuugc gaugcaaaaa ggugcugacg acacaucgaa acggugaaag 60
ccguaggucc   70

<210> 140
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 140
gggcgacccu gaugagucgg gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
auacgaaacg gugaaagccg uagguugcc   89

61

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<210> 141
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: BRK sensitive
      hammerhead nucleic acid sensor molecule

<400> 141
gggcgacccu gaugagggag gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
gcgcgaaacg gugaaagccg uagguugcc                                    89


<210> 142
<211> 87
<212> RNA
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<220>
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<220>
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<220>
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<222> (75)..(77)
<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (79)
<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (81)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (86)
<223> Wherein n is an a or u or c or g or nothing

<400> 142
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gccaaacagn gaaannngna nguggnc                                      87


<210> 143
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
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<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 143
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gaacgaaacg gugaaagccg uagguugcc   89

<210> 144
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
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      hammerhead nucleic acid sensor molecule

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<220>
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<222> (63)
<223> Wherein n is an a or u or c or g or nothing

<400> 144
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acncgaaacg gugaaagccg uagguugcc   89

<210> 145
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 145
gggcgacccu gaugaguugc gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
uaacgaaacg gugaaagccg uagguugcc   89

<210> 146
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 146
gggcgacccu gaugaguguu gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
cuacgaaacg gugaaagccg uagguugcc   89

<210> 147

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<211> 89
<212> RNA
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<220>
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      hammerhead nucleic acid sensor molecule

<220>
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<223> Wherein n is an a or u or c or g or nothing

<220>
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<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (85)
<223> Wherein n is an a or u or c or g or nothing

<400> 147
gggcgacccu gaugaguaau gcuaaggagg auuuccgaaa gcggcucggg ccgccaguua  60
ggccaaacgg ngaaagcccg uangnugcc                                    89


<210> 148
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 148
gggcgacccu gaugagcucu gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua  60
uggccgaaac ggugaaagcc guagguugcc                                   90


<210> 149
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 149
gggcgacccu gaugaggcau gcuaaggagg auuuccgaaa gcggcuacgg uccgccagug  60
cggcgaaacg gugaaagccg uagguugcc                                    89


<210> 150
<211> 90
<212> RNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 150
gggcgacccu gaugagcccc gcuaaggagg auuuccgaaa gcggcuacug guccgccagu 60
ugagcgaaac ggugaaagcc guagguugcc   90

<210> 151
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

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<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (73)
<223> Wherein n is an a or u or c or g or nothing

<400> 151
gggcgacccu gaugaguacg gcunaggagg auuuccgaaa gcggnuacug guccgncagu 60
nanacgaaac ngngaaagcc guagguugcc   90

<210> 152
<211> 91
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 152
gggcgacccu gaugagccau gcuaaggagg auuuccgaaa gcggcuacug guccgccagu 60
acuacagaaa cggugaaagc cguagguugc c   91

<210> 153
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 153
gggcgacccu gaugagugac gcuaaggagg auuuccgaaa gcggcuacug guccgccagu 60
ugcgcgaaac ggugaaagcc guagguugcc   90

<210> 154
<211> 90
<212> RNA
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<220>
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      hammerhead nucleic acid sensor molecule

<400> 154
gggcgacccu gaugagucug gcuaaggagg auuuccgaaa gcggcuacug guccgccagu 60
uucgcgaaac ggugaaagcc guagguugcc   90

<210> 155
<211> 90
<212> RNA
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<220>
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accgaaacgg ccuaaagccg gagguugcc   89

<210> 157
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cuagccgaaa cggugaaagc cguagguugu cc   92

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caacgaaacg gugaaagccg uagguugcc   89

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cugcgaaacg gugaaagccg uagguugcc                                   89


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augcgaaacg gugaaagccg uagguugcc                                   89


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<210> 165
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auacgaaacg gugaaagccg uagguugcc   89

<210> 166
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uaacgaaacg gugaaagccg uagguugcc   89

<210> 168
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71

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ucacgaaacg gugaaagccg uagguugcc   89

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acgcgaaacg gugaaagcgu angnugcc   88 .

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aaccgaaacg gugaaagccg uagguugcc   89

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uggcgaaacg gugaaagccg uagguugcc   89

<210> 177
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acugcgaaac ggugaaagcc guagguugcc   90

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aguacgaaac ggugaaagcc guagguugcc                                    90


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aaacgaaacg gugaaagccg uagguugcc   89

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agucgaaacg gugaaagccg uagguugcc                                   89


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<213> Artificial Sequence

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<210> 196
<211> 89
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<212> RNA
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gcgcgaaacg gugaaagccg uagguugcc                                   89


<210> 197
<211> 89
<212> RNA
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agacgaaacg gugaaagccg uagguugcc   89

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cuacgaaacg gugaaagccg uagguugcc   89

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aaacgaaacg gugaaagccg uagguugcc   89

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<210> 242
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (34)
<223> Wherein n is an a or u or c or g or nothing

<400> 242
gggcgacccu gaugagcuca gcuaaggagg auunccgaaa gcggcuacgg uccgccaguu 60
ucgcgaaacg gugaaagccg uagguugcc                                  89


<210> 243
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (46)
<223> Wherein n is an a or u or c or g or nothing
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<400> 243
gggcgacccu gaugaggcuu gcuaaggagg auuuccgaaa gcggcnacgg uccgccaguc 60
cgucgaaacg gugaaagccg uagguugcc   89

<210> 244
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 244
gggcgacccu gaugagcucu gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
cggcgaaacg gugaaagccg uagguugcc   89

<210> 245
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 245
gggcgacccu gaugaggcaa gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
caacgaaacg gugaaagccg uagguugcc   89

<210> 246
<211> 82
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 246
gggcgacccu gaugagcguc gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
ucucgaaacg gugaaagccg ua   82

<210> 247
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 247
gggcgacccu gaugagcuuc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
aaacgaaacg gugaaagccg uaggu   85

<210> 248
<211> 75
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 248
gggcgacccu gaugaguggc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
uaacgaaacg gugaa   75

<210> 249
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 249
gggcgacccu gaugagacuc gcuaaggagg auuuccgaaa gcggcuacgg uccgccagug 60
guacgaaacg gugaaagccg uaggu   85

<210> 250
<211> 91
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (35)
<223> Wherein n is an a or u or c or g or nothing

<400> 250
gggcgacccu gaugaguauu gcuaaggagg auuunccgaa agcggcucac ggugccgcca 60
guugagcgaa acggugaaag ccuagguugc c   91

<210> 251
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (18)
<223> Wherein n is an a or u or c or g or nothing

101

<400> 251
gggcgacccu gaugagcnuc gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
auacgaaacg gugaaagccg uagguugc   88

<210> 252
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<220>
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<222> (19)..(20)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (62)
<223> Wherein n is an a or u or c or g or nothing

<400> 252
gggcgacccu gaugagcunn gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
cnacgaaacg gugaaagccg uagguugcc   89

<210> 253
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 253
gggcgacccu gaugaguucg gcuaaggagg auuuccgaaa gcggcuacgg uccgccagug 60
aagcgaaacg gugaaagccg uaggu   85

<210> 254
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 254
gggcgacccu gaugagauga gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
aaccgaaacg gugaaagccg uagguugcc   89

<210> 255
<211> 77
<212> RNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (40)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (44)
<223> Wherein n is an a or u or c or g or nothing

<400> 255
gggcgacccu gaugagaauc gcuaaggagg auuuccgaan gcgncuacgg uccgccaguu 60
ccccgaaacg gugaaag   77

<210> 256
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (62)
<223> Wherein n is an a or u or c or g or nothing

<400> 256
gggcgacccu gaugagucac gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
gncgcgaaac ggugaaagcc guagguugcc   90

<210> 257
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 257
gggcgacccu gaugagcauc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
aagcgaaacg gugaaagccg uagguugcc   89

<210> 258
<211> 84
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive

hammerhead nucleic acid sensor molecule

<400> 258
gggcgacccu gaugagguca gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
cagcgaaacg gugaaagccg uagg   84

<210> 259
<211> 79
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 259
gggcgacccu gaugagaagc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguc 60
uagcgaaacg gugaaagcc   79

<210> 260
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 260
gggcgacccu gaugaggcuu gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
gaucgaaacg gugaaagccg ugagguugcc   90

<210> 261
<211> 91
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 261
gggcgacccu gaugaggcug gcuaaggagg auuuccgaaa gcggcucacg guccgccagu 60
ccuaacgaaa cggugaaagc cguagguugc c   91

<210> 262
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 262
gggcgacccu gaugagccuu gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
agucgaaacg gugaaagccg uagguugc   88

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<210> 263
<211> 67
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (61)
<223> Wherein n is an a or u or c or g or nothing

<400> 263
gggcgacccu gaugagucac gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
ncgcgaa                                                           67


<210> 264
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 264
gggcgacccu gaugagaacu gcuaaggagg auuuaccgaa agcggcuacg guccgccagu 60
ugcgcgaaac ggugaaagcc guagguugcc                                  90


<210> 265
<211> 84
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 265
gggcgacccu gaugagcgac gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
gggcgaaacg gugaaagccg uagg                                        84


<210> 266
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 266
gggcgacccu gaugaguaac gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguc 60
agacgaaacg gugaaagccg uaggu                                       85
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<210> 267
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 267
gggcgacccu gaugaggacu gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
acucgaaacg gugaaagccg uaggu   85

<210> 268
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 268
gggcgacccu gaugagccgg gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
gagcgaaacg gugaaagccg uagguugcc   89

<210> 269
<211> 41
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (19)
<223> Wherein n is an a or u or c or g or nothing

<400> 269
gggcgacccu gaugagcana gcuaaggagg auuuccgaaa g   41

<210> 270
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 270
gggcgacccu gaugaguugc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
uagcgaaacg gugaaagccg uaggu   85

<210> 271
<211> 85
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 271
gggcgacccu gaugagcagc gcuaaggagg auuuccgaaa gcggcuaggu ccgccagucu 60
ucgaaacggu gaaagccgua gguug 85

<210> 272
<211> 75
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 272
gggcgacccu gaugaguaag gcuaaggagg auuuccgaaa gcggcuacgg uccgccagug 60
uugcgaaacg gugaa 75

<210> 273
<211> 89
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 273
gggcgacccu gaugagcacc gcuaaggagg auuuccgaaa gcggcuacgg uccgccaguu 60
acgcgaaacg gugaaagccg uagguugcc 89

<210> 274
<211> 82
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
     hammerhead nucleic acid sensor molecule

<400> 274
gggcgacccu gaugaggcau gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
aggcgaaacg gugaaagccg ua 82

<210> 275
<211> 77
<212> RNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 275
gggcgacccu gaugaguuua gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
gaccgaaacg gugaaag                                                  77


<210> 276
<211> 77
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 276
gggcgacccu gaugagaagc gcuaaggagg auuuccgaaa gcggcuacgg uccgccagua 60
augcgaaacg gugaaag                                                  77


<210> 277
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (46)
<223> Wherein n is an a or u or c or g or nothing

<400> 277
gggugaccuc ugauag+uguu gcuaaggagg gauuuccgaa agcggnuacg guccgccagu 60
agagcgaaac ggugaaagcc guagguugcc                                   90


<210> 278
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (9)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (19)
<223> Wherein n is an a or u or c or g or nothing
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<220>
<221> misc_feature
<222> (46)
<223> Wherein n is an a or u or c or g or nothing

<400> 278
gggcgaccnu gaugagcang cgcuaaggag gauuuccgaa agcggnuacg guccgccagu 60
gacucgaaac ggugaaagcc guagguugcc                                  90


<210> 279
<211> 80
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (33)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (70)..(71)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (76)
<223> Wherein n is an a or u or c or g or nothing

<400> 279
gggcgacccu gauaguagcg cuaaguaagg auuuccgaa agcgcgcuca cgguccgcca 60
gucucacgan ncggunaaag                                             80


<210> 280
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (4)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (20)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
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<222> (52)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (65)
<223> Wherein n is an a or u or c or g or nothing

<400> 280
gggngacccu gaugaguaun agcuaaggag gauuuccgaa agcggcuacg gnccgccagu 60
aaugngaaac ggugaaagcc guagguugcc                                 90


<210> 281
<211> 90
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (19)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (23)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (46)
<223> Wherein n is an a or u or c or g or nothing

<400> 281
gggcgaccuu gaugaaggnc agnuaaggag gauuuccgaa agcggnuacg gucagccagu 60
ggcacgaaac ggugaaagcc guagguugcc                                 90


<210> 282
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<400> 282
gggcgacccu gaugagcaug cuaaggagga uuuccgaaag cggcuacggu ccgccaguua 60
gucgaaacgg ugaaagccgu agguugcc                                   88


<210> 283
<211> 87
<212> RNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence: ERK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (16)..(17)
<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (22)
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<220>
<221> misc_feature
<222> (40)
<223> Wherein n is an a or u or c or g or nothing

<220>
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<222> (56)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (60)..(61)
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<220>
<221> misc_feature
<222> (72)
<223> Wherein n is an a or u or c or g or nothing

<220>
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<220>
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<222> (81)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (86)
<223> Wherein n is an a or u or c or g or nothing

<400> 283
gggcgacccu gauganngcu gnuaggagga uuuccgaaan ugcuucuggc ccgcanuuan 60
nccgaacggg cnaaancgaa nggugnc   87

<210> 284
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive

hammerhead nucleic acid sensor molecule

<400> 284
gggcgacccu gaugaguagg cuaaggagga uuucgaaagc ggcuacgguc cgccagugug 60
ccgaa   65

<210> 285
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: BRK sensitive
      hammerhead nucleic acid sensor molecule

<220>
<221> misc_feature
<222> (18)
<223> Wherein n is a or u or c or g or nothing.

<220>
<221> misc_feature
<222> (25)
<223> Wherein n is a or u or c or g or nothing.

<220>
<221> misc_feature
<222> (50)
<223> Wherein n is a or u or c or g or nothing.

<220>
<221> misc_feature
<222> (60)
<223> Wherein n is a or u or c or g or nothing.

<400> 285
gggcgacccu gcugagcnac gcuangagga uuuccgaaag cggcuacggn ccgccagucn 60
gacgaaacgg ugaaagccgu agguugcc   88

<210> 286
<211> 87
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 1-14

<400> 286
gggcgacccu gaugaggguc gcuaaggagg auuuccgaaa gcggcacggu ccgccagacg 60
ucgaaacggu gaaagccgua gguugcc   87

<210> 287
<211> 87
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 1-13

<400> 287
gggcgacccu gaugagucuu cuaaggagga uuuccgaaag cggcacgguc cgccaguacg 60
ucgaaacggu gaaagccgua gguugcc   87

<210> 288
<211> 86
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 1-2

<400> 288
gggcgacccu gaugagccuu cuaaggagga uuuccgaaag cggcacgguc cgccagacgu 60
cgaaacggug aaagccguag guugcc   86

<210> 289
<211> 87
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 1-6

<400> 289
gggcgacccu gaugagucau gcuaaggagg auuuccgaaa gcggcacggu ccgccagacg 60
ucgaaacggu gaaagccgua gguugcc   87

<210> 290
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-7

<400> 290
gggcgacccu gaugagccuu gcuaaggagg auuuccgaaa gcggcacggu ccgccaguca 60
gucgaaacgg ugaaagccgu agguugcc   88

<210> 291
<211> 87
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-2

<400> 291
gggcgacccu gaugagccuc gcuaaggagg auuuccgaaa gcggcacggu ccgccagcua 60
gcgaaacggu gaaagccgua gguugcc   87

<210> 292
<211> 88
<212> RNA

113

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-3

<400> 292
gggcgacccu gaugagaacu gcuaaggagg auuuccgaaa gcggcacggu ccgccaguuu 60
aacgaaacgg ugaaagccgu agguugcc   88

<210> 293
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-13

<400> 293
gggcgacccu gaugaguaaa gcuaaggagg auuuccgaaa gcggcacggu ccgccagucu 60
ggcgaaacgg ugaaagccgu agguugcc   88

<210> 294
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-14

<400> 294
gggcgacccu gaugaguuuu gcuaaggagg auuuccgaaa gcggcacggu ccgccaguga 60
uccgaaacgg ugaaagccgu agguugcc   88

<210> 295
<211> 88
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 2-20

<400> 295
gggcgacccu gaugaggcga gcuaaggagg auuuccgaaa gcggcacggu ccgccaguuu 60
aacgaaacgg ugaaagccgu agguugcc   88

<210> 296
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Oligonucleotide substrate

<220>
<223> Description of Artificial Sequence: 5' primer

114

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<400> 296
tctaatacga ctcactatag gacctcggcg aaagc                          35


<210> 297
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 3' Primer A

<400> 297
actctcgcta acctctctag tcata                                     25


<210> 298
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 3' Primer B

<400> 298
agtgctctcg cacctaacct ctctagt                                   27


<210> 299
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 3' Primer C

<400> 299
agtgcgagcc tctcggctcg cacctaacct ctctagt                        37


<210> 300
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #1 from
      first set

<400> 300
tctaatacga ctcactatag gacctcggcg aaagc                          35


<210> 301
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #2 from
      first set
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<400> 301
agtgctctcg cacctaacct ctctagt                                    27


<210> 302
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer 1 from
      second set

<400> 302
gttgctacaa atgatatgac                                            20


<210> 303
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #2 from
      second set

<400> 303
atggcaattt agccatgaga                                            20


<210> 304
<211> 106
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Ligase RNA
      sequence

<400> 304
ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uaggugcauc uucaugucca gucgcuugca augccc               106


<210> 305
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Reverse
      transcriptase primer

<400> 305
gggcattgca agcgactgga cat                                        23


<210> 306
<211> 28
<212> DNA
```

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide substrate

<400> 306
actgaacctg accgtacaaa gatgcacu   28

<210> 307
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TaqMan forward
      primer

<400> 307
actgaacctg accgtacaaa ga   22

<210> 308
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TaqMan reverse
      primer

<400> 308
tttgtagcaa catggcaatt ta   22

<210> 309
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TaqMan probe
      primer

<400> 309
cggcgaaagc taacgtctca tgg   23

<210> 310
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Forward primer

<400> 310
actgaacctg accgtacaaa ga   22

```
<210> 311
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Reverse primer

<400> 311
tttgtagcaa catggcaatt ta                                          22


<210> 312
<211> 95
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Lysozyme
      dependent nucleic acid sensor molecule

<400> 312
ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uaggugccuc gugaugucca gucgc                            95


<210> 313
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector oligo

<400> 313
gcgactggac atcacgag                                               18


<210> 314
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Substrate

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide substrate

<400> 314
gtacgatgcg atgctagcga ttgttgugca cu                               32


<210> 315
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
```

<223> Description of Artificial Sequence: Forward primer

<400> 315
taatacgact cactatagga cttcggcgaa agccgttcga cc   42

<210> 316
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer
      spanning the 3' proximal portion of the L1 ligase
      domain

<400> 316
attcgagatg tccttggacc aaagccgcac ctaacctcct gtctaag   47

<210> 317
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Forward primer

<400> 317
gcgaccttac gatcagatga c   21

<210> 318
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Reverse primer

<400> 318
ccgcacctaa cctcctgtct aa   22

<210> 319
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK ligase
      probe primer

<400> 319
aaggaggatt tccgaaagcg gctacg   26

<210> 320
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK ligase
      probe primer

<400> 320
cgctagcgaa ttggttcctc gaaagg   26

<210> 321
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Substrate

<220>
<223> Description of Artificial Sequence: Substrate 3

<400> 321
catgcgacct tacgatcaga tgacctugca cu   32

<210> 322
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Substrate 3
      specific 5' PCR primer

<400> 322
catgcgacct tacgatcaga t   21

<210> 323
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Regeneration
      5' PCR primer

<400> 323
tctaatacga ctcactatag gacttcggcg aaagc   35

<210> 324
<211> 109
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Library (DNA)
      (random regions 3-5 nucleotides in length)

<220>

```
<221> misc_feature
<222> (26)..(30)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (64)..(68)
<223> Wherein n is an a or t or c or g or nothing

<400> 324
ggacttcggc gaaagccgtt cgaccnnnnn aaggaggatt tccgaaagcg gctacggtcc 60
gccnnnnnct tagacaggag gttaggtgcg taggtaaccg atagttccg            109


<210> 325
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector
      primer

<400> 325
cggaactatc ggttacctac                                           20


<210> 326
<211> 135
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      selection-specific oligonucleotides

<220>
<221> misc_feature
<222> (26)..(31)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (89)..(94)
<223> Wherein n is an a or t or c or g or nothing

<400> 326
ggacttcggc gaaagccgtt cgaccnnnnn ncagacgcta gcgaattggt tcctcgaaag 60
gggaaagcgt tattaagaaa ccaaaatgnn nnnncttaga caggaggtta ggtgcgtcaa 120
tgctgcaagt tactg                                                135


<210> 327
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector
      primer
```

```
<400> 327
cagtaacttg cagcattgac                                          20


<210> 328
<211> 101
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: bFGF selection
     specific oligonucleotide

<220>
<221> misc_feature
<222> (26)..(32)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (54)..(60)
<223> Wherein n is an a or t or c or g or nothing

<400> 328
ggacttcggc gaaagccgtt cgaccnnnnn nngcaacgct acagacaagt gcannnnnnn 60
cttagacagg aggttaggtg cccgagttgt tcgaacgaga c                   101


<210> 329
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector
     primer

<400> 329
gtctcgttcg aacaactcgg                                           20


<210> 330
<211> 92
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Thrombin
     selection specific oligonucleotide

<220>
<221> misc_feature
<222> (26)..(31)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (47)..(51)
<223> Wherein n is an a or t or c or g or nothing

<400> 330
```

```
ggacttcggc gaaagccgtt cgaccnnnnn natcgaagtt agtaggnnnn ncttagacag 60
gaggttaggt gcgtcaatcg attgcagatc cg                                 92
```

```
<210> 331
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Effector
      primer

<400> 331
cggatctgca atcgattgac                                                 20
```

```
<210> 332
<211> 77
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nucleic acid
      sensor molecule with effector domain deleted

<400> 332
ggaccucggc gaaagcuaac gucucauggc uaaauugcca uguugcuaca aaugauauga 60
cuagagaggu uaggugc                                                   77
```

```
<210> 333
<211> 83
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: MK.08.92.A

<400> 333
gcttgcaagc ccttagaccc tgatgagcct tgcgatgcaa aaaggtgctg acgacacatc 60
gaaacggtga aagccgtagg tct                                            83
```

```
<210> 334
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: MK.08.66.B

<400> 334
agacctacgg ctttcaccgt ttcg                                            24
```

```
<210> 335
<211> 57
<212> DNA
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence: MK.08.130.B

<400> 335
atacgactca ctataggatg tccagtcgct tgcaatgccc ttttagaccc tgatgag   57

<210> 336
<211> 82
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      LYS.LIG.TMPLT.L1-11.2

<400> 336
ctataggact tcggcgaaag ctaacgtctc atggctaaat tgccatgttg ctacaaatga 60
tatgactaga gaggttaggt gc   82

<210> 337
<211> 92
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      FMN.LIG.TMPLT.L1-R7C13

<400> 337
ctataggact tcggtccagt gctcgtgcac taggccgttc gaccttcagg atatgcttcg 60
gcagaaggga acttagacag gaggttaggt gc   92

<210> 338
<211> 101
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      THEO.LIG.TMPLT.L1-D1

<400> 338
ctataggact tcggtccagt gctcgtgcac taggccgttc gaccatgata ccagcatcgt 60
cttgatgccc ttggcagcat cttagacagg aggttaggtg c   101

<210> 339
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TK.16.32.A

<400> 339
ttctaatacg actcactata ggacttc   27

```
<210> 340
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TK.16.32.B

<400> 340
attcgagatg tccttggacc aaagccgcac ctaacctc                      38


<210> 341
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Substrate

<220>
<223> Description of Artificial Sequence: TK.16.32.15NT

<400> 341
attcgagatg tccttggacc aaagcctcca tcgtgcgcac ctaacctc          48


<210> 342
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Substrate

<220>
<223> Description of Artificial Sequence: TK.04.82.A

<400> 342
catgcgacct tacgatcaga tgacctugca cu                            32


<210> 343
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: MK.08.125A

<400> 343
tccatcgtgc gcacu                                              15


<210> 344
<211> 27
<212> DNA
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence: TK16.32.A

<400> 344
ttctaatacg actcactata ggacttc   27

<210> 345
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: MK.08.125B

<400> 345
tccatcgtgc gcacctaacc tc   22

<210> 346
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: MK.08.125A

<400> 346
tccatcgtgc gcacu   15

<210> 347
<211> 55
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
      Oligonucleotide substrate

<220>
<223> Description of Artificial Sequence: 2-piece ligase
      template

<220>
<221> misc_feature
<222> (26)..(33)
<223> Wherein n is an a or u or c or g or nothing

<400> 347
ggacuucggc gaaagccguu cgaccnnnnn nnncuuagac aggagguuag gugcg   55

<210> 348
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide substrate

<400> 348
tccatcgtgc gcacu　　　　　　　　　　　　　　　　　　　15

<210> 349
<211> 99
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 29

<400> 349
ggacuucggc gaaagccguu cgaccacgcu aaggaggauu uccgaaagcg gcuacgugcc 60
gccagugucu uagacaggag guuaggugcg cacgaugga　　　　　　　99

<210> 350
<211> 6
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
　　　Oligonucleotide substrate

<400> 350
ugcacu　　　　　　　　　　　　　　　　　　　　　　　　6

<210> 351
<211> 97
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Construct 30

<400> 351
ggacuucggc gaaagccguu cgaccagcua aggaggauuu ccgaaagcgg cuacgugccg 60
ccagcucuua gacaggaggu uaggugcgca cgaugga　　　　　　　97

<210> 352
<211> 138
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
　　　dependent ligase template

<220>
<221> misc_feature
<222> (27)..(30)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (87)..(90)

127

<223> Wherein n is an a or u or c or g or nothing

<400> 352
ggacuucggc gaaagccguu cgaccannnn cagacgcuag cgaauugguu ccucgaaagg 60
gaaagcguua uuaagaaacc aaaaugnnnn ucuuagacag gagguuaggu gccguccgac 120
ugaucucgga guuaaacg   138

<210> 353
<211> 107
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK
      sensitive hammerhead template

<220>
<221> misc_feature
<222> (17)..(21)
<223> Wherein n is an a or u or c or g or nothing

<220>
<221> misc_feature
<222> (76)..(80)
<223> Wherein n is an a or u or c or g or nothing

<400> 353
gggcgacccu gaugagnnnn nagacgcuag cgaauugguu ccucgaaagg ggaaagcguu 60
auuaagaaac caaaannnnn cgaaacggug aaagccguag guugccc   107

<210> 354
<211> 134
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ppERK nucleic
      acid sensor molecule template

<220>
<221> misc_feature
<222> (25)..(31)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (88)..(93)
<223> Wherein n is an a or t or c or g or nothing

<400> 354
ggacttcggc gaaagccgtt cgaccnnnnn ncagacgcta gcgaattggt tcctcgaaag 60
ggaaagcgtt attaagaaac caaaatgnnn nnncttagac aggaggttag gtgcgtcaat 120
gctgcaagtt actg   134

<210> 355
<211> 109
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ERK sensitive
      nucleic acid sensor molecule template

<220>
<221> misc_feature
<222> (26)..(30)
<223> Wherein n is an a or t or c or g or nothing

<220>
<221> misc_feature
<222> (64)..(68)
<223> Wherein n is an a or t or c or g or nothing

<400> 355
ggacttcggc gaaagccgtt cgaccnnnnn aaggaggatt tccgaaagcg gctacggtcc 60
gccnnnnnct tagacaggag gttaggtgcg taggtaaccg atagttccg   109

<210> 356
<211> 131
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CW45-33-A08
      ERK sensitive nucleic acid sensor molecule

<220>
<223> Description of Combined DNA/RNA Molecule:
      Oligonucleotide substrate

<400> 356
ggacuucggc gaaagccguu cgacccucuc agacgcuagc gaauugguuc cucgaaaggg 60
aaagcguuau uaagaaacca aaaugagagg cuuagacagg agguuaggug cgucaaugcu 120
gcaaguuacu g   131

<210> 357
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide substrate

<400> 357
acgtagcata gcatcgatag ctgttgugca cu   32

<210> 358
<211> 265
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Thymidylate
      synthase ("td") intron

```
<400> 358
taattgaggc ctgagtataa ggtgacttat acttgtaatc tatctaaacg gggaacctct 60
ctagtagaca atcccgtgct aaattgtagg actgcccggg ttctacataa atgcctaacg 120
actatccctt tggggagtag ggtcaagtga ctcgaaacga tagacaactt gctttaacaa 180
gttggagata tagtctgctc tgcatggtga catgcagctg gatataattc cggggtaaga 240
ttaacgacct tatctgaaca taatg                                      265


<210> 359
<211> 276
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Theophylline
      dependent td group I intron

<400> 359
taattgaggc ctgagtataa ggtgacttat acttgtaatc tatctaaacg gggaacctct 60
ctagtagaca atcccgtgct aaattgatac cagcatcgtc ttgatgccct tggcagcata 120
aatgcctaac gactatccct ttggggagta gggtcaagtg actcgaaacg atagacaact 180
tgctttaaca agttggagat atagtctgct ctgcatggtg acatgcagct ggatataatt 240
ccggggtaag attaacgacc ttatctgaac ataatg                         276


<210> 360
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Exon 1, the
      5'-exon

<400> 360
tttcttgggt                                                       10


<210> 361
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Exon 2, the
      3'-exon

<400> 361
ctaccgttta                                                       10


<210> 362
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 362
agtgctctcg cacctaacct cctgtct                                    27
```

```
<210> 363
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 363
ggacttcggc gaaagc                                                16


<210> 364
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 364
agtgctctcg cacctaacct cctgtct                                    27


<210> 365
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 365
gctacggtcc gccagttctt                                            20


<210> 366
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 366
cgctttcgga atcctccctt                                            20


<210> 367
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 367
gctacggtcc gccaggggct                                            20
```

```
<210> 368
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 368
cgctttcgga aatcctcctt                                                    20


<210> 369
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 369
gctacggtcc gccaaaagct                                                    20


<210> 370
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 370
cgctttcgga aatcctcctt                                                    20


<210> 371
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 371
aaggggaaag cgttattaag                                                    20


<210> 372
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 372
tcgaggaacc aattcgctag                                                    20
```

(54) Title: NUCLEIC ACID SENSOR MOLECULES AND METHODS OF USING SAME

(57) Abstract: Methods for engineering a nucleic acid sensor molecule are provided. Biosensors comprise a plurality of nucleic acid sensor molecules labeled with a first signaling moiety and a second signaling moiety. The nucleic acid sensor molecules recognizes target molecules which do not naturally bind to DNA. Binding of a target molecule to the sensor molecules triggers a change in the proximity of the signaling moieties which leads to a change in the optical properties of the nucleic acid sensor molecules on the biosensor. Reagents and systems for performing the method are also provided. The method is useful in diagnostic applications and drug optimization.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/25319

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :   C12Q1/68; C07H 21/02, 21/04
US CL   :   435/6; 536/23.1, 24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 536/23.1, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,589,332 A (SHIH et al) 31 December 1996, see at least for example Figure 1C and Column 5 at the paragraph beginning at about line 45.	1-2, 8-11, 37-40, 44-45, 59, and 61-62 ---------- 3-7, 41-43, 46-58, and 63-71
X --- Y	WO96/27026 A1 (INTELLIGENE LTD.) 06 September 1996, see entire document	1-11, 37-40, 44, 59, 61-62 ---------- 41-43, 45-58, 60, and 63-71
A	US 6,201,113 B1 (TODD et al) 13 March 2001, see the entire document.	1-11 and 37-71

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 May 2003 (01.05.2003)	17 JUL 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Ethan Whisenant, Ph.D. *Janice Ford* Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO99/47704 A1 (JENNE et al) 23 September 1999, see the entire document.	1-11 and 37-71
A	HASELOFF et al. Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature. 18 August 1988, Vol. 334, pages585-591, see the entire document.	1-11 and 37-71
A	WO99/50277 A1 (UNIVERSITY OF UTAH RESEARCH FOUNDATION) 07 October 1999, see the entire document.	1-11 and 37-71
A	TUSCHL et al. A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements. Science. 04 November 1994, Vol. 266, pages 785-789, see the entire document.	1-11 and 37-71

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/25319

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-11 and 37-71

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING
Lack of Unity

1. This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group 1, Claim(s) 1-11 and 37-71 drawn to a nucleic acid sensor molecule which molecule comprises a catalytic domain which catalytic domain comprises a *cis*-endonucleolytic ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 2, Claim(s) 1-11 and 37-69 drawn to a nucleic acid sensor molecule which molecule comprises a catalytic domain which catalytic domain comprises a *trans*-endonucleolytic ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 3, Claim(s) 1-8, 12-14, 37-73, 76, 78-86, 93 94 and 97, drawn to a nucleic acid sensor molecule which is a self-ligating 1-piece *cis*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 4, Claim(s) 1-8, 12-14, 37-73, 94, and 97, drawn to a nucleic acid sensor molecule which is a self-ligating 1-piece *trans*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 5, Claim(s) 1-8, 12-14, 37-73, 95, and 98, drawn to a nucleic acid sensor molecule which is a self-ligating 2-piece *cis*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 6, Claim(s) 1-8, 12-14, 37-73, 95, and 98, drawn to a nucleic acid sensor molecule which is a self-ligating 2-piece *trans*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 7, Claim(s) 1-8, 12-14, 37-73, 96, and 99, drawn to a nucleic acid sensor molecule which is a self-ligating 3-piece *cis*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 8, Claim(s) 1-8, 12-14, 37-69, 96 and 99, drawn to a nucleic acid sensor molecule which is a self-ligating 3-piece *trans*-ligase ribozyme and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 9, Claim(s) 1-8, 15-16, 18, 21, 23, 37-74, 78-88, 89 and 90, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is a protein kinase and wherein said protein kinase is ERK1 or ERK2 and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 10, Claim(s) 1-8, 15-16, 18, 29 and 37-73, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is a protein kinase and wherein said protein kinase is RAF kinase and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 11, Claim(s) 1-8, 15-16, 18, 32 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is is a GTP binding protein and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 12, Claim(s) 1-8, 15-16, 18, 31 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is is a phosphatase and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 13, Claim(s) 1-8, 15-16, 18, 31, 34 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is a cytokine and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 14, Claim(s) 1-8, 15-16, 30, and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is a Ras protein and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 15, Claim(s) 1-8, 15-16, 18, 31, 33 and 37-69 drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a protein, wherein said protein is GPCR and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 16, Claim(s) 1-8, 15-17, and 37-69 drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes post-translationally modified form of a protein wherein the post-translational modification is selected from a defined group which includes prenylation, glycosylation, methionine removal, N-acetylation, acylation acylation of cysteines, myristolylation, alkylation, ubiquitinylation, prolyl-4-hydroxylation, carboxylation of glutaminyl residues, advanced glycosylation, deamination of glutamine and asparagine, addition of glycophosphatidylinositol, disulfide bond formation, hydroxylation and lipidation; and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 17, Claim(s) 1-8, 15-17, 19-20, 22, 37-69, 75, 78-86, and 91, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a post-translationally modified form of a protein wherein the post-translational modified protein is phosphorylation and wherein said phosphorylation is monophosphorylation or diphosphorylated and wherein said post-translationally modified diphosphorylated protein is pp ERK and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 18, Claim(s) 1-8, 15, 34 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes peptides and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **19**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes nucleic acids and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **20**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes oligosaccharides and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **21**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes nucleotides and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **22**, Claim(s) 1-8, 15 and 36-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes metabolites and/or cellular metabolites and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **23**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes drugs and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **24**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes toxins and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **25**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes biohazards and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **26**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes ions and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **27**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes carbohydrates and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **28**, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes polysaccharides and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group **29**, Claim(s) 1-8, 15, and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes hormones and

composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 30, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes receptors and wherein said receptor is a GPCR and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 31, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes antigens and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 32, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes antibodies and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 33, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a virus and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 34, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a co-factor and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 35, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a dye and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 36, Claim(s) 1-8, 15 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a nutrient and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 37, Claim(s) 1-8, 15, 35 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a growth factor and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 38, Claim(s) 1-8, 24-28 and 37-69, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes a component, product, or associated protein/product of a MAP kinase pathway and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

Group 39, Claim(s) 1-8, 37-69, 77-86, and 92, drawn to a nucleic acid sensor molecule which comprises a target modulation domain which recognizes a target selected from a defined group which includes cCMP, cAMP,

or cGMP pathway and composition(s) thereof, as well as, a method of using the nucleic acid sensor molecule defined above in this paragraph.

2. The inventions listed as Groups I-39 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical feature(s).

The claims as drawn are related to each other because of the product, i.e. the nucleic acid sensor molecule. However, since the the nucleic acid sensor molecule as claimed (Claim 1) was known - see, for example, Shih et al. US 5,589,332 (31 DEC 1996), see at least Figure 1a of Shih et al.- the claims are no longer linked by a special technical feature, because, by definition, the special technical feature must distinguish over the prior art. Without the special technical feature the claims lack unity.

3. In addition, if the applicant elects to (i.e. pays to) have any of Groups 3-9, 17 or 39 searched the applicant will also be required to elect a single nucleotide sequence for searching from each of Claims 89-93 and 97-99.

Continuation of B. FIELDS SEARCHED Item 3:
USPATFULL via East, Medline CAplus
searcbh terms: probe?, cis-endonucleolytic adj ribozyme?, ribozyme?, FRET